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A MEDIUM FOR PERFUSION OF INNERVATED BLOOD VESSELS

BY

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(RECEIVED JULY 6, 1953)

Interest in drugs which lower blood pressure has increased with the use of these agents in treating human hypertension. To determine whether such drugs act by causing vasodilatation as the result of peripheral vascular action, perfused blood vessel preparations are usually employed. By perfusing limbs or organs which retain only nervous connexions with the remainder of the animal it is possible to examine on the perfused vessels not only the direct effects of drugs but also effects mediated by the nervous system.

Homologous blood is almost the only medium at present suitable for such perfusions, but, although it preserves the reactivity of the nervous connexions, it has disadvantages. The most important of these is that, for reasons of economy, it is usually necessary to recirculate the blood, so that any drug tested remains in the perfusate and may interfere with subsequent tests. Furthermore, mechanical trauma inseparable from recirculation and reoxygenation of the blood may lead to haemolysis, with the liberation of vasoactive substances (Chambliss, Demming, Wells, Cline, and Eckstan, 1950 ; Binet and Burstein, 1950).

This paper reports the use of a perfusion medium which maintains nervous reactivity, but which is without some of the disadvantages of homologous blood.

Preparation and Properties of Perfusion Medium

The medium consists of a suspension of red blood cells in Krebs–Henseleit Ringer solution containing 6% dextran.

Both human and sheep red cells have been used. The former were the residue from the preparation of plasma, and the latter were obtained from citrated sheep's blood recently collected at the slaughterhouse. The use of red cells from a different species does not seem to interfere with the vascular reactivity of the preparation in short-term experiments, provided that the cells are washed free of citrated plasma. This was done by centrifuging 250 ml. of cells in 1 litre of 0.9% (w/v) NaCl for 30 min. After a second wash with saline there was a final wash with the Ringer solution of Krebs and Henseleit (1932), buffered with bicarbonate to *p*H 7.4. The washed packed red cells were then suspended in Krebs–Henseleit solution containing 6% dextran (average mol. wt. 75,000) to give a packed cell volume of 40%. The medium was oxygenated by bubbling oxygen containing 5% CO_2 through it. After equilibration, the *p*H was checked with a calomel and glass electrode *p*H meter.

Oxygen Capacity and Availability.—The oxygen capacity of six samples oxygenated in this way was measured by a microvolumetric technique to be reported elsewhere (Saunders, unpublished); it averaged 18 vol. % with a range of 15–20 vol. %. The oxygen seemed to be readily available, since the venous effluent was considerably darker than the medium before perfusion.

Oedema Formation.—If the venous return remained unobstructed, no oedema of skin or muscle was observed in experiments lasting up to 3 hr. In the absence of dextran, however, oedema occurred within 15–30 min. Unlike other colloids, such as gum acacia, dextran does not appear to contain vasoconstrictor substances; no vasodilatation was produced by ACh (10 μg.) injected into the perfusate after the nervous tone had been removed by section of the nervous connexions (Fig. 1).

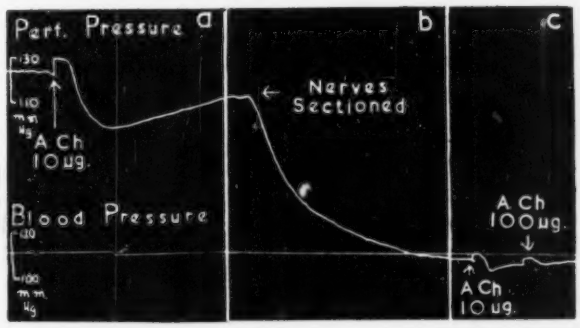

FIG. 1.—Rat, hind quarters perfused independently; simultaneous records of arterial blood pressure and perfusion pressure. (a) ACh 10 μg. injected into perfusate, followed by (b) division of nerve supply to limb, (c) ACh 10 and 100 μg. injected into perfusate.

Maintenance of Vascular Reactivity to Nervous Influences.—The hind-limbs of rabbits or the hind-quarters of rats, isolated from the rest of the circulation but with intact nervous connexions, were perfused by the method of Fastier and Smirk (1947). In this, the perfusion fluid is passed to the arterial supply of the perfused area by a constant-output rotary pump. Variation in the peripheral resistance gives rise to alterations in pressure which are recorded on a smoked drum with a Hg manometer.

It was consistently possible to demonstrate the presence of peripheral vascular tone of nervous origin in the perfused limb vessels. Section of the nerve supply to the limb was always followed by vasodilatation. For the maintenance of nervous reactivity it seems important that the *p*H should be kept between 7.2 and 7.4; alterations beyond this range resulted in a rapid loss of demonstrable tone from the perfused area.

The preservation of nervous reactivity was demonstrated by the injection of test substances into the general circulation of the animal. Raising or lowering the systemic blood pressure with intravenous injections of adrenaline or ACh gave rise to changes in the perfusion pressure of the perfused limb. When the systemic pressure of the rat or rabbit was raised by adrenaline, there was a fall in the perfusion pressure, whereas the fall of systemic blood pressure elicited by ACh was associated with a rise in the peripheral vascular tone of the perfused limb. Since the perfused area was isolated from the rest of the body except for nervous connexions, the changes in perfusion pressure produced by adrenaline or ACh could be mediated only through the nervous system.

Asphyxia, produced by clamping the trachea of rats, caused a rise in systemic blood pressure accompanied by a rise in the vascular tone of the perfused limb.

Use of Medium in Investigation of Vasodilator Actions

Results obtained with hexamethonium bromide illustrate the utility of the perfusion medium for the study of depressor agents of this type. Six experiments were performed on rabbits with the perfused hind-limb having only nervous connexion with the rest of the body. Hexamethonium bromide (1 to 5 mg.) dissolved in 0.1 ml. of Krebs–Henseleit solution at *p*H 7.4 did not produce vasodilatation in the perfused limb when injected into the fluid entering the perfusion cannula, whereas ACh (10 μg.) produced vasodilatation with a fall of perfusion pressure of 40 to 60 mm. Hg when given by the same route. When hexamethonium bromide (10 to 15 mg.) was injected intravenously, however, there was a fall of systemic blood pressure of about 40 mm. and a simultaneous fall in perfusion pressure of from 60 to 80 mm. Hg (Fig. 2). The fall in perfusion pressure was presumably

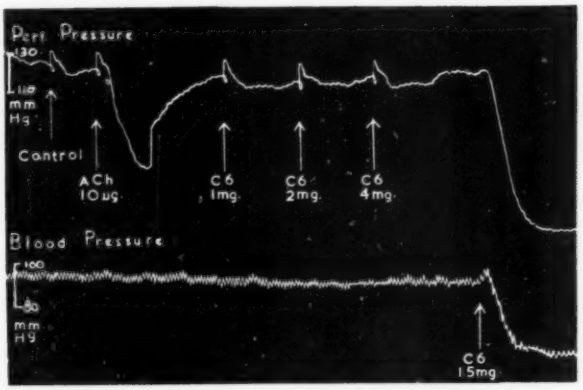

FIG. 2.—Rabbit, hind-limb perfused independently; simultaneous records of arterial blood pressure and perfusion pressure: Injection of ACh (10 μg.) and of hexamethonium bromide (1, 2, and 4 mg. C_6) into perfusate; injection of hexamethonium bromide intravenously (15 mg. C_6).

due to a decrease in nervous tone, since subsequent section of the nerves did not produce any additional fall in perfusion pressure.

Summary

1. A new perfusion medium is described. It is a suspension of washed human or sheep red cells in Krebs–Henseleit Ringer solution containing 6% dextran.

2. It preserves nervous reactivity in perfused isolated innervated blood vessel preparations, and may therefore facilitate the study of the actions of hypotensive drugs.

The author wishes to acknowledge the encouragement given by Professor F. H. Smirk. The expenses were defrayed in part by the Medical Research Council of New Zealand. The dextran was generously supplied by Messrs. Bengers, Ltd.; Crookes Laboratories, Ltd.; Glaxo Laboratories, Ltd.; and Messrs. Salmond and Spraggon, Ltd.

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Brit. J. Pharmacol. (1954), **9**, 131.

THE INHIBITION OF THE PERISTALTIC REFLEX BY SYMPATHOMIMETIC AMINES

BY

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(RECEIVED AUGUST 21, 1953)

The peristaltic reflex initiated by raising the pressure in the lumen of the isolated intestine of rabbits and guinea-pigs (Trendelenburg's method) is inhibited by low concentrations of adrenaline and noradrenaline (McDougal and West, 1952). The nature of the mechanism by which this inhibition is produced is still somewhat uncertain. Adrenaline could either relax the muscle fibres directly, or interfere with transmission at various levels in the peristaltic reflex arc—at, for instance, the ganglionic synapse or the neuromuscular junction.

It seemed of interest to investigate the inhibition of the peristaltic reflex further, by determining the effect of various sympathomimetic amines upon it, and whether the effects of these are influenced by sympatholytic drugs. Such observations might be expected to throw light on the mechanism whereby the reflex is inhibited.

METHODS

Strips of guinea-pig ileum (2–3 cm. long) were suspended in aerated Tyrode's solution at 37° C. in a 50 ml. isolated organ bath. The intestinal volume was recorded by the method of Trendelenburg (1917) as modified by Feldberg and Solandt (1942). The peristaltic reflex was initiated by raising the pressure in the lumen by 2–3 cm. of Tyrode's solution.

In other experiments, similar strips of ileum were suspended in aerated Tyrode's solution at 37° C. in a 15 ml. bath, and the longitudinal muscle contractions were recorded with a frontal writing lever (magnification ×8). Preparations of strips of rabbit ileum and uterus were also tested.

The sympathomimetic amines used are listed in Table I, the sympatholytic drugs in Table III, and other drugs in Table IV.

RESULTS

Activities of Various Sympathomimetic Amines

Peristaltic Inhibitory Action.—Concentrations of 10^{-8} adrenaline regularly produce inhibition of the peristaltic reflex in the isolated guinea-pig gut (Fig. 1). The activities of the other amines relative to that of adrenaline are shown in Table I, each value being the geometric mean of at least three experiments. The most potent compounds are all dihydroxyphenylalkylamines. The dihydroxyphenyl compounds studied are shown in Table II, which gives their structures, and, for comparison, their relative activities in stimulating the isolated rabbit uterus and in inhibiting the pendular movements of the isolated rabbit ileum (geometric means of four experiments). It is evident that in the more potent amines a relationship exists between these three effects, though notable exceptions are isoprenaline (compound 10), α-ethylnoradrenaline (9), and adrenalone (7). Isoprenaline does not possess any excitor action on the rabbit uterus.

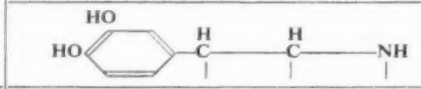
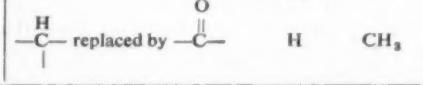
TABLE I

RATIO OF DOSES OF SYMPATHOMIMETIC AMINES AND AMINO-ACIDS TO EQUIACTIVE DOSE OF (−)-ADRENALINE ON GUINEA-PIG ILEUM

No.	Amine	Inhibition of Peristalsis	Inhibition of Nicotine Contraction	No. of Expts.
1	(−)-Adrenaline	1	1	30
2	(−)-Noradrenaline	4	2	20
3	(±)-Corbasil	10	5	3
4	Epinine	17	15	3
5	(±)-*N*-Ethylnoradrenaline	20	8	3
6	Lactyladrenaline	20	20	3
7	Adrenalone	28	20	3
8	Lactylnoradrenaline	40	35	3
9	(±)-α-Ethylnoradrenaline	86	30	3
10	(±)-Isoprenaline	200	200	20
11	Hydroxytyramine	250	100	3
12	Tyramine	1,200	1,000	3
13	Amphetamine	1,200	560	6
14	Ephedrine	1,300	540	6
15	Dihydroxyphenylalanine	1,500	1,500	3
16	Dihydroxyphenylserine	3,000	3,000	3
17	Propadrine	3,000	500	3
18	*m*-Sympatol	5,000	1,000	3
19	Sympatol	6,000	2,000	3
20	*m*-Norsynephrine	6,000	2,000	3
21	Paredrine	6,000	1,000	3
22	*p*-Norsynephrine	7,500	2,000	3

TABLE II

RELATIONSHIP BETWEEN STRUCTURE AND ACTIVITY OF DIHYDROXYPHENYL COMPOUNDS, THE ACTIONS OF WHICH ON THE GUINEA-PIG PERISTALTIC REFLEX AND ON RABBIT UTERUS (BUT NOT ON RABBIT ILEUM) ARE PREVENTED BY SYMPATHOLYTIC DRUGS

Amine No.	Formula	Ratio of Dose to Equiactive Dose of Adrenaline		
		Peristaltic Reflex (Guinea-pig)	Rabbit Uterus (Excitor)	Rabbit Ileum (Inhibitor)
2	OH H H	4	1	2
1	OH H CH_3	1	1	1
5	OH H C_2H_5	20	15	8
10	OH H $CH(CH_3)_2$	200	—	20
3	OH CH_3 H	10	30	10
9	OH C_2H_5 H	86	500	100
16	OH COOH H	3,000	>1,000	>10,000
11	H H H	250	200	200
4	H H CH_3	17	30	25
15	H COOH H	1,500	>1,000	>10,000
7		28	500	40

Interaction with Motor Drugs.—The results of experiments on the inhibition of the nicotine response of the longitudinal muscle of the guinea-pig ileum are also recorded in Table I. The concentrations of the amines and amino-acids required to reduce the nicotine response have little effect on equally strong contractions produced by ACh or by histamine (see Fig. 2A). An ACh, but not a nicotine, contraction is maintained, for example, in the presence of 1 in 50,000 adrenaline ; relaxation of the nicotine contraction starts 5 sec. after adding the adrenaline to the bath.

Feldberg and Lin (1949) showed that, when the peristaltic reflex in the isolated rabbit gut is abolished by cocaine or hexamethonium, eserine (10^{-7} to 10^{-6}) elicits rhythmic contractions affecting the whole muscle simultaneously. They concluded that the circular muscle is still responsive to eserine although the nervous elements are depressed. We repeated this experiment with most of the sympathomimetic amines and obtained similar results on adding eserine in their presence. It is, however, easier to obtain this effect with dihydroxyphenylalkylamines than with the other amines.

These results show that the inhibitory action of the dihydroxyphenylalkylamines on peristalsis is not due to a diminution in the sensitivity of the muscle fibres to motor drugs in general, and, more specifically, to ACh. They suggest, rather, that this effect of adrenaline, and of its derivatives, is due to an interruption of the peristaltic reflex arc, either by virtue of the known ganglion-blocking action of the amines, or by their suppressing the release of ACh.

Sympatholytic Drugs

Effect on Peristaltic Inhibitory Action of Dihydroxyphenylalkylamines.—It is well known that

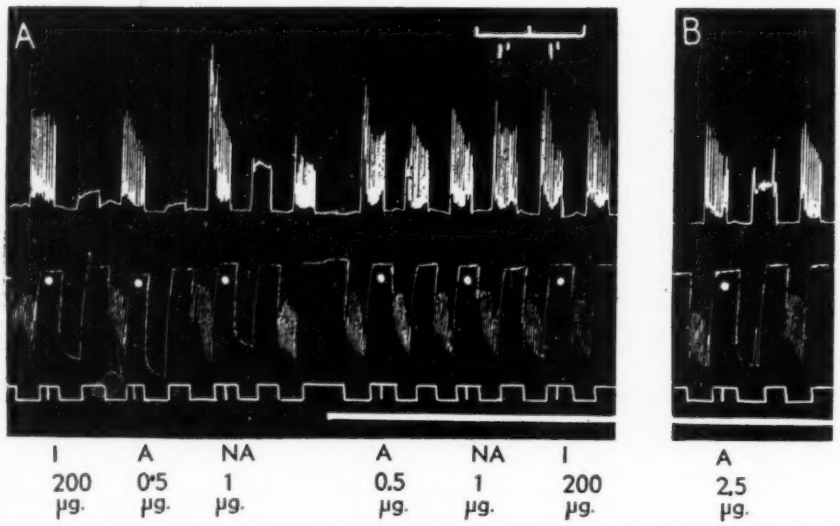


FIG. 1.—Isolated guinea-pig ileum. Bath volume 50 ml. Top tracing, contractions of longitudinal muscle; middle tracing, intestinal volume; lower tracing, marker indicating (downwards) rise of pressure in the lumen, and points of injection of drugs. Time in min. In A, Adrenaline (A, 0.5 µg.), noradrenaline (NA, 1 µg.) and isoprenaline (I, 200 µg.) inhibit the peristaltic reflex. These doses, however, are ineffective in the continuous presence of 933F (10^{-7}), indicated by the white line. In B, increasing the dose of adrenaline to 2.5 µg. overcomes this block.

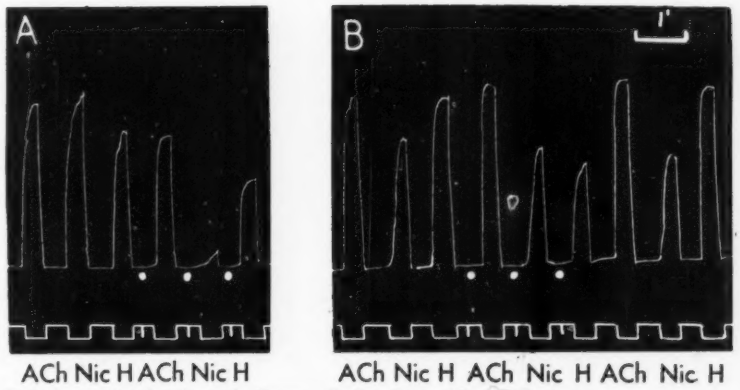


FIG. 2.—Isolated guinea-pig ileum. Bath volume 15 ml. Contractions of longitudinal muscle. Time in min. In A, effects of acetylcholine (ACh, 0.2 μg.), nicotine (Nic, 20 μg.) and histamine (H, 0.2 μg.) alone, and in the presence of adrenaline, 1 μg. (indicated by the dots). In B, the same drugs in the continuous presence of 933F (10^{-7}).

anti-adrenaline (sympatholytic) drugs block most excitatory actions of adrenaline (e.g., that on the rabbit uterus) but fail to suppress most of its inhibitory actions (e.g., that on the muscle fibres of the rabbit ileum). In the presence of sympatholytic drugs, however, the action of all the dihydroxyphenylalkylamines on the peristaltic reflex of the guinea-pig is prevented (Fig. 1). This result suggests that the anti-peristaltic action of these amines is exerted on nervous structures and not directly on the muscle fibres. Effective concentrations of the blocking drugs are recorded in Table III, together with their concentrations

TABLE III

CONCENTRATION OF SYMPATHOLYTIC DRUGS REQUIRED TO ANTAGONIZE THE ACTIONS OF DIHYDROXYPHENYLALKYLAMINES ON THE PERISTALTIC REFLEX OF THE GUINEA-PIG AND ON THE RABBIT UTERUS

Drug	Peristaltic Reflex	Rabbit Uterus	Type of Antagonism	Concn. of Sympatholytic Drug which Itself Inhibits Peristalsis
Tolazoline ..	5×10^{-7}	2×10^{-6}	Reversible	2×10^{-4}
933F ..	10^{-7}	5×10^{-7}	,,	4×10^{-5}
Dibenamine..	2×10^{-7}	2×10^{-7}	Complete	2×10^{-6}
SKF 688A ..	5×10^{-8}	5×10^{-8}	,,	10^{-6}
SY 28 ..	2×10^{-8}	2×10^{-8}	,,	10^{-6}

needed to antagonize the amine response on the rabbit uterus (an excitor action). There appears to be a close similarity in the effective concentrations of the blocking drugs for these two effects. Whereas the action of tolazoline and 933F is reversible (the block is easily washed away or may be overcome by increasing the dose of the amine, as shown in Fig. 1), that of the other three related drugs is complete (the block is not removed by washing and cannot be overcome by increasing the dose, as shown in Fig. 3). High doses of the sympatholytic drugs themselves inhibit peristalsis (Table III), although this may be the result of some depressant action on the nervous or muscular elements.

Effect on Nicotine-stimulation.—On the longitudinal muscle of the guinea-pig ileum, the inhibition of the nicotine contraction by dihydroxyphenylalkylamines is removed when the blocking drugs are present (Fig. 2B).

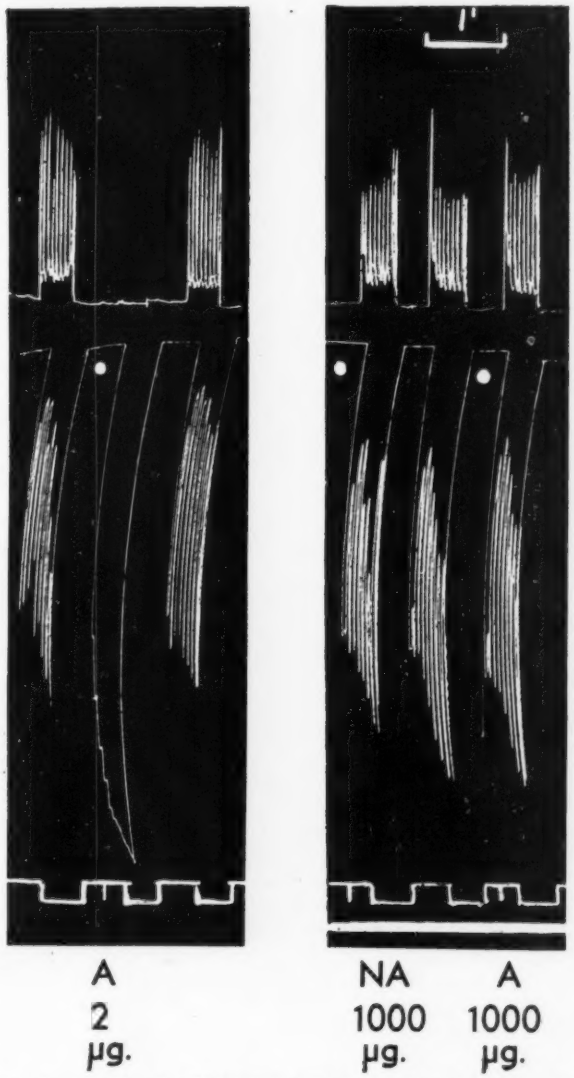


FIG. 3.—As Fig. 1. White line indicates continuous presence of dibenamine (2×10^{-7}). Note that the action of this sympatholytic agent in removing inhibition is complete, and cannot be overcome by increasing the dose of adrenaline (A) or noradrenaline (NA).

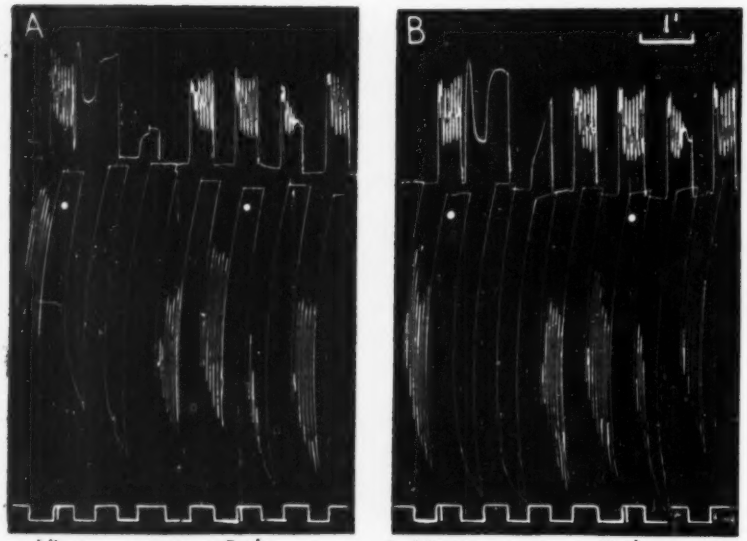


FIG. 4.—As Fig. 1. In A, the effect of nicotine (Nic, 600 μg.) and pethidine (Peth, 50 μg.) on the peristaltic reflex. In B, these effects in the continuous presence of 933F (10^{-7}).

This action is easier to obtain with 933F or tolazoline than with one of the other water-insoluble blocking drugs.

TABLE IV

CONCENTRATION OF DRUGS WHICH INHIBIT THE PERISTALTIC REFLEX OF THE GUINEA-PIG ILEUM AND ARE UNAFFECTED BY SYMPATHOLYTIC DRUGS

Drug	Concn.
Hexamethonium	10^{-5}
Nicotine	10^{-5}
(+)-Tubocurarine	10^{-5}
Cocaine	5×10^{-6}
Atropine	10^{-8}
Pethidine	10^{-6}
Mepyramine	2×10^{-5}

Effect on the Peristaltic Inhibition Produced by Other Drugs.—To see if sympatholytic agents can counteract other types of peristaltic inhibition, we have examined their effect on the inhibition produced by ganglion-blocking agents (hexamethonium, nicotine and (+)-tubocurarine), by drugs known to have little ganglionic blocking action (atropine, pethidine, and mepyramine), and by a local anaesthetic (cocaine). Table IV gives the concentrations of these drugs needed to inhibit the peristaltic reflex in the guinea-pig ileum. Sympatholytic agents do not affect the action of any of this varied group of drugs (Fig. 4).

These results show that the nature of the inhibitory action of the dihydroxyphenylalkylamines on peristalsis differs radically from that of the other substances enumerated, and that the antagonism by sympatholytic drugs is restricted specifically to the site of action of the sympathomimetic amines.

Monohydroxyphenyl- and Phenyl-alkylamines

These amines are listed in Table V. The importance of the *meta*-hydroxyphenyl group for stimulation of the rabbit uterus and relaxation of the rabbit ileum is shown (compare the activities of compounds 18 and 20 with those of compounds 19 and 22 respectively). Yet all of these compounds are almost inactive in suppressing the peristaltic reflex in the guinea-pig. Since there is no correlation with the other two effects, the inhibition of the reflex by the compounds shown in

TABLE V

RELATIONSHIP BETWEEN STRUCTURE AND ACTIVITY OF AMINES, THE ACTIONS OF WHICH ON THE PERISTALTIC REFLEX AND RABBIT ILEUM ARE NOT PREVENTED BY SYMPATHOLYTIC DRUGS

Amine No.	R^1	R^2	R^3	R^4	R^5	Ratio of Dose to Equiactive Dose of Adrenaline: Peristaltic Reflex (Guinea-pig)	Rabbit Uterus (Excitor)	Rabbit Ileum (Inhibitor)
12	OH	H	H	H	H	1,200	1,000	—
21	OH	H	H	CH_3	H	6,000	1,000	—
22	OH	H	OH	H	H	7,500	500	4,000
19	OH	H	OH	H	CH_3	6,000	100	2,000
20	H	OH	OH	H	H	6,000	20	50
18	H	OH	OH	H	CH_3	5,000	5	10
13	H	H	H	CH_3	H	1,200	>1,000	>10,000
17	H	H	OH	CH_3	H	3,000	200	>10,000
14	H	H	OH	CH_3	CH_3	1,300	1,000	>10,000

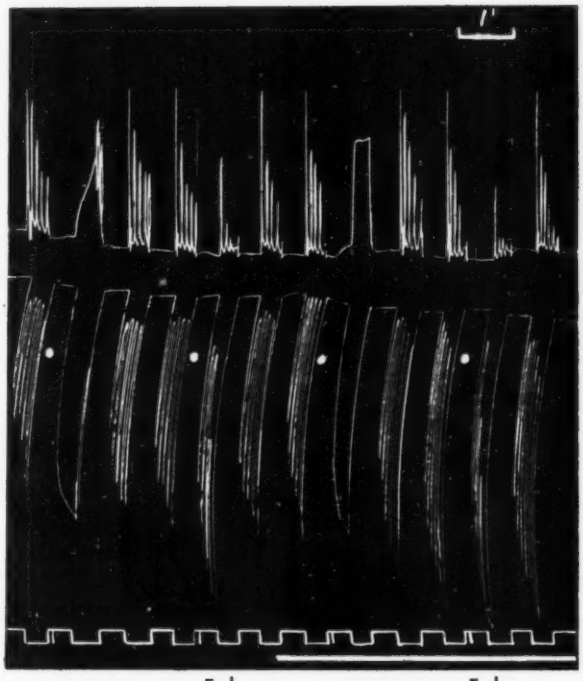


FIG. 5.—As Fig. 1. White line indicates continuous presence of 933F (10^{-7}). Note that the action of meta-sympatol (m-sym, 10.0 mg.), and of ephedrine (Eph, 2.5 mg.), is uninfluenced by this concn. of 933F.

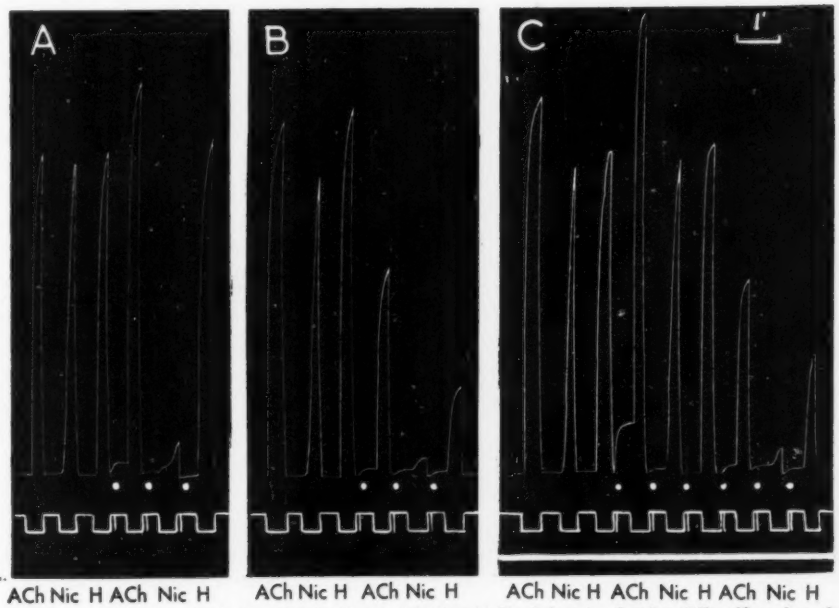


FIG. 6.—As Fig. 2. In A, the effects of acetylcholine (ACh, 0.4 μg.), nicotine (Nic, 40 μg.) and histamine (H, 0.4 μg.) alone and in the presence of adrenalone 10 μg. (indicated by the dots). In B, the same drugs alone and in the presence of amphetamine 500 μg. (indicated by the dots). In C, the white line indicates the continuous presence of 933F (10^{-7}); the first three dots indicate the presence of adrenalone (10 μg.); the second three, amphetamine (500 μg.). For further explanation, see text.

Table V may be of a non-specific nature. This is supported by the failure of sympatholytic drugs to suppress the effect of these compounds on the peristaltic reflex and on the nicotine response of the longitudinal muscle, although the excitation of the rabbit uterus by these amines is abolished. Fig. 5 illustrates this point on the peristaltic reflex, the actions of a monohydroxyphenyl compound (meta-sympatol) and an unsubstituted phenyl compound (ephedrine) being uninfluenced by the continuous presence of 933F. Fig. 6 illustrates the comparison on the longitudinal muscle for adrenalone on the one hand (inhibition of the nicotine response prevented), and for amphetamine on the other (inhibition not affected). It is noticeable in the tracing that both adrenalone and amphetamine exert a weak motor action which is not suppressed by the sympatholytic drugs.

DISCUSSION

We have shown that a series of 22 sympathomimetic amines inhibit the peristaltic reflex and reduce the nicotine contraction of the longitudinal muscle of the guinea-pig ileum without greatly affecting those of histamine and ACh. Among the more active members of the series there is some correlation between the relative ability to produce these two effects and some other well-known actions of the amines—such as stimulation of the rabbit uterus and relaxation of the rabbit ileum—but this is not so with the less active ones. When the peristaltic reflex is inhibited, eserine produces rhythmic contractions and an increase in tone of the circular muscle, as it does in the presence of ganglion-blocking concentrations of hexamethonium (Feldberg and Lin, 1949; Feldberg, 1951). Since the muscle is also still responsive to ACh, it is probable that these sympathomimetic amines block or depress ganglionic transmission.

A direct depressant action of adrenaline on the response of the superior cervical ganglion in the dog to repetitive stimulation of its preganglionic trunk has been reported by Marrazzi (1939).

It was then considered that adrenaline may block ACh by acting at the same site as the latter, since the action was found to be independent of the vasoconstriction produced. Bülbring and Burn (1942) also demonstrated this ganglion-blocking action by experiments in dogs in which the ganglia of the lumbar sympathetic chain and the vessels of the hind leg were separately perfused. Small concentrations of adrenaline, added to the blood perfusing the ganglia, increased the vasoconstrictor effect of preganglionic stimulation, but higher concentrations reduced the effect to zero. They also noted than in atropinized spinal cats the ganglionic stimulant action of ACh was augmented by small doses, but depressed by large doses, of adrenaline. Paton and Thompson (1953) have recently shown that in the superior cervical ganglion of the cat adrenaline not only diminishes the release of ACh in response to preganglionic stimulation but also depresses the action of ACh on ganglion cells.

Why should adrenaline and noradrenaline depress the activity of parasympathetic ganglia? Such a depression would reduce the excitor effects of preganglionic parasympathetic stimulation in the gut, thereby augmenting the relaxant action of these amines on the effector muscle cells at the postganglionic sympathetic nerve endings. Since there is now strong evidence that transmission at the parasympathetic ganglionic synapse is cholinergic (Perry and Talesnik, 1953), adrenaline must antagonize the action of ACh at this site. The antagonism, however, is not of the same type as that with hexamethonium or (+)-tubocurarine (competitive antagonism), which is unaffected by sympatholytic drugs. Nicotine block, which is said to result from depolarization of the ganglion cells, is also unchanged by sympatholytic drugs; thus the mechanism by which adrenaline and the related amines produce ganglionic blockade is not clear, although it appears to be related either to the phenomena described by Marrazzi (1939) and by Bülbring and Burn (1942), or to those of Paton and Thompson (1953) and of Ellis (1953).

We have found that sympatholytic drugs specifically prevent the inhibition of peristalsis by dihydroxyphenylalkylamines and that they fall into two groups—firstly, one where the block is reversible and easily removed by washing (tolazoline and 933F), and secondly, one where the block is complete (the β-chlorophenylethylamines). In both groups the concentrations required for this effect are very similar to those required to prevent stimulation of the rabbit uterus. Sympatholytic drugs, however, do not influence the action of the sympathomimetic amines on the isolated rabbit ileum. Thus there is evidence that the ganglionic blocking action of the dihydroxyphenylalkylamines may be classed with excitor effects. But there is the difference that both the *meta*- and *para*-hydroxy groups in the ring are most important for inhibition of peristalsis, whereas the *meta*-hydroxy group is the most important for stimulation of the excitor receptors in the rabbit uterus.

We have also found that many of the dihydroxyphenylalkylamines excite the lower third of the guinea-pig ileum (as reported by Munro, 1951). All the sympatholytic drugs used block this excitation in the same concentrations as are required to prevent the inhibition of the peristaltic reflex. This is of interest, since Munro (1952) has shown that this action of adrenaline is probably a direct one on the smooth muscle.

It is also interesting that the catechol ring has already been shown to be essential for various other ganglionic and neuromuscular effects; for example, augmentation of the response to ACh in the isolated perfused superior cervical ganglion of the cat (Konzett, 1950), potentiation of the twitch tension developed by the tibialis anterior muscle in the cat when stimulated indirectly (Huidobro, Cubillos, and Eyzaguirre, 1952), and decurarization in the nerve-muscle preparation of the dog (Maddock, Rankin, and Youmans, 1948).

When we turn to the monohydroxyphenylalkylamines and the phenylalkylamines, we find three important facts: (1) the doses needed to inhibit the reflex are large, since all are more than 1,000 times less active than adrenaline, (2) the relative activities do not correspond either with those on the rabbit uterus or on the rabbit ileum, and (3) sympatholytic drugs do not influence this action. These observations imply that the effects of the compounds on the reflex may be non-specific. However, other drugs which act specifically on the nervous components of the reflex are also unaffected by sympatholytic agents.

We consider the evidence sufficient to state that adrenaline, noradrenaline, and other dihydroxyphenylalkylamines have an action on the parasympathetic ganglia in the guinea-pig ileum. Adrenaline, noradrenaline, and other dihydroxyphenylalkylamines have what Ahlquist (1948) would have called an α-effect on the parasympathetic ganglia in the guinea-pig ileum. This response is altered by sympatholytic agents as specifically as the excitor actions of sympathomimetic amines (with the exception of that on the heart).

Summary

1. Dihydroxyphenylalkylamines are potent inhibitors of the peristaltic reflex in the guinea-pig ileum. The mechanism by which this inhibition is produced is not clear, although it is probably a direct depressant action on transmission in the ganglionic synapses within the enteric plexus.

2. The contraction of the longitudinal muscle produced by nicotine is likewise reduced by doses of the amines which have little or no effect on the contractions produced by histamine and acetylcholine.

3. Sympatholytic agents prevent these two actions of the dihydroxyphenylalkylamines without influencing those of hexamethonium and of nicotine, but do not antagonize the block produced by hexamethonium and by paralytic doses of nicotine.

4. Monohydroxyphenylalkylamines and phenylalkylamines are feeble in these actions; they are not affected by sympatholytic drugs.

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SOME SELECTIVE INHIBITORS OF TRUE CHOLINESTERASE*

BY

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While studying certain diphenyl pentanones as possible neuromuscular blocking agents, it was found that 1:5-bis(4-trimethyl-ammoniumphenyl)-*n*-pentan-3-one diiodide (62C47, Wellcome) had a marked selective inhibitory action on "true cholinesterase." The distribution of this type of activity was therefore investigated in further members of this and analogous series. The general formula of the compounds is

wherein R_1 and R_2 are usually substituted quaternary nitrogens of varying degrees of complexity, and A is usually either CO or CHOH (Table I). Copp (1953) has described their chemical properties. The present paper deals with some of their pharmacological actions.

METHODS

Cholinesterase Inhibition.—Anticholinesterase activity was determined manometrically at 37° C. in the Warburg apparatus (Ammon, 1933) with 0.025 M-$NaHCO_3$ as medium equilibrated with 5% CO_2 and 95% N_2. Rat brain, homogenized in 15 parts by weight of 0.025 M-$NaHCO_3$, was the source of the true enzyme and horse serum that of pseudo-cholinesterase. The enzyme preparation—1 ml. rat brain homogenate or 0.3 ml. horse serum—was introduced into the main compartment of the bottle together with a fresh solution of the inhibitor dissolved in 0.025 M-$NaHCO_3$. The substrate, also dissolved in 0.025 M-$NaHCO_3$, was placed in the side arm. The total fluid volume, adjusted by the addition of 0.025 M-$NaHCO_3$, was always 3.0 ml. The final substrate concentrations were 0.003 M or 0.012 M-acetylcholine (bromide) when testing true cholinesterase activity and 0.008 M-benzoylcholine (iodide) when testing pseudo-cholinesterase inhibition. Enzyme and inhibitor were in contact for 25 min. while gaseous and thermal equilibria were being established, before the substrate was added. All the results were corrected for non-enzymic hydrolysis of the substrate, and the inhibitor values were calculated from the results obtained after equilibrium had been established between enzyme, substrate and inhibitor.

Action on Nerve-Muscle Preparations.—The rat diaphragm–phrenic nerve preparation (Bülbring, 1946) was used to investigate (*a*) the ability to increase the response to indirect stimulation by square pulses of 0.34 msec. duration, (*b*) antagonism to the paralysing action of (+)-tubocurarine chloride by the method of Mogey and Young (1949), and (*c*) paralysing activity. This last was compared directly against, and expressed as a percentage of, the activity of (+)-tubocurarine in the way described by Mogey, Trevan, and Young (1949) except that, instead of allowing drugs to act for a standard period, time was given for equilibrium to be reached. The compounds were added at intervals until activity was shown or a concentration of $10^{-3.5}$ had been obtained. The stimuli used were square wave impulses of maximal intensity, and of 0.34 msec. duration, applied once every 12 seconds.

In vivo paralysing activity was determined for some of the compounds in the cat anaesthetized with pentobarbitone sodium. The response of the gastrocnemius to sciatic nerve stimulation was recorded by a spring lever and the drugs were injected into the femoral vein of the other leg.

Toxicity.—The drugs were administered intravenously in saline to mice, each mouse receiving 0.5 ml. solution per 20 g. body weight.

Other methods, not requiring detailed description, are referred to in the most appropriate places in the text.

RESULTS

Cholinesterase Inhibition.—Table I gives estimates of the activity against the cholinesterase of rat brain (true) and that of horse serum (pseudo), expressed as *p*I 50 values (*p*I 50 = the negative logarithm of the molar concentration causing 50% inhibition). Whereas all the results reported for the activity against true cholinesterase were from experiments where 0.012 M-acetylcholine was used, some experiments were done with 0.003 M-acetylcholine. The degree of inhibition was usually greater with the lower substrate concentration. This indicates that at the higher concentrations the acetylcholine did not itself cause inhibition and

* The gist of this paper formed a communication (by Gertrude E. Glock and G. A. M.) to the British Pharmacological Society in Edinburgh, July, 1948: a few members of the series were included in a demonstration (by J. W. Trevan and G. A. M.) to the Society at Beckenham, January, 1948.

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so increase the apparent effectiveness of the drugs: it also suggests that the inhibition is of a competitive nature. Many workers condemn the use of substrate concentrations greater than the optimal when dealing with true cholinesterase. At low substrate concentrations, however, the results are less reliable than at the high, because the total CO_2 evolved is much less and it is difficult to maintain CO_2 production for a time sufficient to demonstrate the establishment of equilibrium before the substrate is completely hydrolysed.

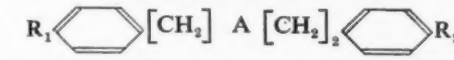
In addition, to exclude the possible effects of variation of the enzyme source, compounds 62C47, 25C48, eserine, and neostigmine were tested by using a single rat brain as the source of the true cholinesterase. The same results were obtained as when different rat brains were used. As with other cholinesterase inhibitors, atropine sulphate did not inhibit the activity of 25C48 *in vitro*: this was the only compound tested, but it was one of the most potent anticholinesterases.

Since these compounds are cholinesterase inhibitors, it was expected that their injection into mice would increase the toxicity of acetylcholine. The degree of increase would depend on the dose of the inhibitor given, and the time it was in the body before the acetylcholine was injected. Only the most potent inhibitors were examined, and Table II shows some of the results. Detailed analysis of the experiments, including observations at earlier times than those given in the table, showed that most of the compounds came into action about the same time as eserine and more rapidly than neostigmine; that 25C48, 297C50, and 298C50 were about equipotent at the time of maximal effect, and that the duration of the effect of equipotent doses was always nearly the same.

The activity of some of these compounds in increasing the response of the rat diaphragm to indirect stimulation, by square pulses, serves as further evidence of their resemblance to eserine and neostigmine. This increased response, probably due to the setting up of a repetitive discharge at the motor end plate, was abolished by concentrations of (+)-tubocurarine too low to affect the natural twitch response to short stimuli. Atropine antagonized this effect when used in doses large enough to prevent the naturally occurring repetitive responses to long stimuli. Compounds 62C47,

TABLE I

THE COMPOUNDS AND THEIR *p*I 50 VALUES ON TRUE AND PSEUDO-CHOLINESTERASE



Wellcome Code No.	R_1	R_2	A	*p*I 50	
				Rat Brain: Acetylcholine 0·012M	Horse Serum: Benzoylcholine 0·008M
71C48	NMe_3I	NMe_3I	CH_2	6·1	3·6
62C47	NMe_3I	NMe_3I	CO	6·7	3·2
25C48	NMe_2EtI	NMe_2EtI	CO	7·7	<3·0
297C50	NMe_2AllI	NMe_2AllI	CO	7·8	<3·0
298C50	NMe_2nPrI	NMe_2nPrI	CO	7·8	<3·0
312C50	NMe_2nBuBr	NMe_2nBuBr	CO	5·9	3·5
19C51	NMe_2CH_2PhCl	NMe_2CH_2PhCl	CO	5·3	4·8
455C50	$NMeEt_2I$	$NMeEt_2I$	CO	7·5	<3·0
153C47	NMe_3I	H	CO	4·0	3·0
26C48	NMe_3I	OMe	CO	4·6	3·2
143C48	NMe_3I	NMe_3I	CHOH	5·7	<3·0
101C48	NMe_3I	OMe	CHOH	3·9	<3·0
316C50	NMe_2EtI	NMe_2EtI	CHOH	5·9	<3·0
495C50	$NMeEt_2I$	$NMeEt_2I$	CHOH	6·3	<3·0
95C48	NMe_3I	NMe_3I	C_2H_5COH	5·0	<3·0
72C48	NMe_3I	NMe_3I	Ph.COH	4·5	3·3
142C48	NMe_3I	NMe_3I	HCOCOPh	5·6	5·0
Eserine				6·5	8·0
Neostigmine				6·6	7·6

Me=methyl, Et=ethyl, All=allyl, *n*Pr=*n*-propyl, *n*Bu=*n*-butyl, Ph=phenyl. The dibromide corresponding to 297C50 is 284C51.

TABLE II

TOXICITY OF 10 MG./KG. ACETYLCHOLINE BROMIDE INTRAVENOUSLY IN MICE AFTER INTRAPERITONEAL INJECTION OF CHOLINESTERASE INHIBITORS

(This dose of acetylcholine given alone kills 2% of mice)

Inhibitor	Dose (mg./kg.)	Mortality at		
		30 min.	60 min.	120 min.
62C47 ..	1·3	9/10	1/10	0/10
25C48 ..	0·3	8/10	8/10	0/10
	0·15	8/10	3/10	0/10
	0·075	14/20	0/10	
	0·038	9/20		
	0·019	0/10		
297C50 ..	0·075	5/10	0/10	
	0·038	3/10	0/10	2/10
298C50 ..	0·075	5/10	0/10	
	0·038	5/10	1/10	0/10
Neostigmine ..	0·075	19/20	0/10	
	0·038	3/10	0/10	

25C48, 297C50, and 298C50 were almost as active as neostigmine in this respect, whereas 153C47, 26C48, and 95C48 were about one-tenth as active. Compound 72C48 was inactive as far as we could determine. Some of the others showed doubtful activity.

Reversal of Paralysis.—On the rat diaphragm, those compounds most effective in increasing the response to a short stimulus were also very active in opposing the effect of (+)-tubocurarine. The four most potent compounds gave the following figures for the AD 66/33—that is, the concentration in μg./ml. of antagonist which reduces by half the action of a dose of (+)-tubocurarine which by itself would have caused a 66% paralysis (Mogey and Young, 1949) : 62C47, 4.0; 25C48, 1.5; 297C50 and 298C50, 0.1. The less active compounds gave figures of the following order : 153C47, α; 26C48, 105; and 95C48, 78 : compound 72C48, which was relatively inactive in this respect, gave an AD 66/33 of 70. With the lower degrees of activity the AD 66/33 does not appear to give any sensitive differentiation between the members of the various series. As with neostigmine, the antagonism between these compounds and (+)-tubocurarine was not apparent when an excessive dose of the latter was used. The paralysis following (+)-tubocurarine was not reversed by any of the series in the presence of high concentrations of atropine. Because of their structural similarity to decamethonium, 71C48 and 62C47 were examined on a rat diaphragm paralysed by decamethonium. Small quantities of either drug showed no effect, whereas large amounts increased the paralysis.

Paralysing Activity.—The effect of these various compounds on the response of the rat diaphragm-phrenic nerve preparation was studied in considerable detail. As the concentration of the drug was increased, the stimulating effect, already described, gave way to a decrease in the size of the response to a short square impulse (0.34 msec.). We found no evidence of a direct action on the muscle fibre. Phrenic nerve conduction was unaffected by concentrations of 62C47 as high as 10^{-3}. Neostigmine had no effect except in high concentrations, such as $10^{-5.6}$ to $10^{-5.3}$, when it increased the paralysis. Recovery from paralysis was complete after washing and contracture was never induced. 142C48 showed approximately 9% of the paralysing activity of (+)-tubocurarine. The other members of the series showed 6% or less of the activity of (+)-tubocurarine, and most of them 2 or 1%. The degree of specificity of their action on true cholinesterase did not necessarily appear to be a factor of importance in this respect.

With compounds 62C47 and 25C48, the paralysing action was increased by small amounts of (+)-tubocurarine when either long (5.7 msec.) or short (0.34 msec.) stimuli were used. The paralysing effect of 25C48 on the responses to short stimuli was weakly antagonized by catechol ($10^{-5.3}$). Neither decamethonium nor tetramethylammonium iodide antagonized the paralysis after 62C47 or 25C48. Atropine did not reduce the paralysis following any of them. Indeed, the paralysing effect of atropine was easily elicited in the presence of these compounds. When the stimuli to the phrenic nerve were long enough to cause a repetitive discharge, i.e., over 1.25 msec. (Mogey and Trevan, 1948), the paralysing action of atropine appeared at about $10^{-4.3}$. With short stimuli, capable of producing only twitch responses, four or five times as much atropine was required, but, in the presence of paralysing concentrations of 62C47 or 25C48, much smaller concentrations of atropine produced an increase in paralysis.

Increasing the rate of stimulation intensified the block. Tetanizing stimuli (150/sec.) produced either a poorly sustained yet obvious tetanus or a twitch, depending upon the initial degree of paralysis. The twitch was followed by Wedensky inhibition for the duration of the tetanizing stimulus, and when normal rates of stimulation (5/min.) were resumed, there was a temporary reversal of the paralysis. Soon, however, the paralysis was greater than before the tetanus.

The action of adrenaline on the paralysant effect of 25C48 was examined. Small quantities ($10^{-7.5}$ to $10^{-6.5}$) increased the paralysis from 25C48, with either long or short stimuli. After washing 25C48 from the diaphragm, adrenaline produced a marked increase in the size of the response to long or short stimuli.

Some of the compounds were tested on the isolated rectus abdominis of the frog at the temperature of melting ice. 62C47 showed a nicotine stimulating type of action at concentrations from 10^{-6} to $10^{-3.5}$. The other substances showed no such action (26, 71, 101, 142, and 143C48 were not tested). The action of acetylcholine was potentiated with low concentrations of some of the drugs, but was blocked with high concentrations ($10^{-3.5}$) of all of them. Most of the compounds were readily washed from the preparation.

In the cat, compounds 62C47, 153C47, 71C48, 101C48, 297C50, 455C50, 495C50, and 19C51 all caused some paralysis of the gastrocnemius at 0.5 mg./kg. except 71C48, which required 2 mg./

kg., and 153C47, which was still ineffective at 2 mg./kg. Fine muscular fasciculation preceded the paralysis. 0.1 to 0.15 mg./kg. (+)-tubocurarine chloride under these conditions would have caused about 50% paralysis.

Ocular Actions.—With the exception of 101C48, all the drugs were examined on the rabbit eye by direct instillation, and on the mouse eye after intraperitoneal injection of, usually, one-fifth of the intravenous LD50. They all showed varying degrees of miotic effect on parenteral injection, but were inactive on direct application. Compounds 62C47, 25C48, and 71C48 were powerful miotics. Atropine antagonized the miotic effect of 25C48, the only member of the series examined for this action. The effect on intraocular tension was not studied. Compound 312C50, even when instilled in a concentration of 1%, did not produce miosis in the human subject. None of the concentrations up to 1% (in saline) caused any irritation.

Effect on Blood Pressure.—The effect on the blood pressure of the rabbit under pentobarbitone sodium varied from compound to compound. Within the intravenous dose-range 0.17 to 3.2 mg./kg., the commonest effect was a small rise followed by a fall. Similar results were found for most of the series in the anaesthetized cat (pentobarbitone sodium or chloralose). In the atropinized cat, however, 298C50 or 312C50 up to 5 mg./kg. produced no fall.

Toxicity.—The mouse toxicity figures are given in Table III. All the compounds caused symptoms of marked parasympathetic activity—salivation, urination, and defaecation being prominent. Muscular twitchings occurred and sometimes developed into generalized convulsions; after this a flaccid paralysis occasionally appeared. Death seemed to be from respiratory failure: the heart usually continued to beat for a short time after the cessation of respiration. The exhibition of atropine sulphate—20 to 80 mg./kg. intraperitoneally half to two hours in advance, or 2.0 mg./kg. intravenously two minutes before—did not have a significant effect on the toxicity of 25C48, one of the very specific inhibitors. The combined intravenous injection of 0.17 mg./kg. (+)-tubocurarine chloride (approximately the LD50) and 1.3 mg./kg. 25C48 (LD50) was more toxic than either alone: 0.075 mg./kg. (+)-tubocurarine (a non-lethal dose), given simultaneously, had no protective effect against, nor did it increase the toxicity of, 25C48. Indeed, the toxicity was not affected by the previous administration of dibenamine, pentamethonium, nicotine tartrate or a combination of nicotine and atropine.

TABLE III

INTRAVENOUS TOXICITY OF ANTICHOLINESTERASES IN ALBINO MICE

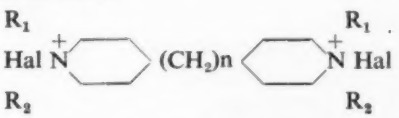
Code No.	LD50 (mg./kg.)	Limits (P=0·95)
71C48	4·2	4·0 –4·4
62C47	2·6	2·5 –2·7
25C48	1·35	1·28–1·41
297C50	2·1	1·96–2·28
298C50	2·0	1·83–2·12
312C50	1·3	1·18–1·41
19C51	0·48	0·42–0·54
455C50	1·0	0·88–1·13
153C47	11·5	10·3 –13·0
26C48	5·8	5·25–6·47
143C48	4·2	3·8 –4·8
101C48	8·25	7·94–8·55
316C50	2·6	2·38–2·86
495C50	1·25	1·07–1·46
95C48	4·4	4·13–4·75
72C48	3·4	3·04–3·75
142C48	1·4	1·3 –1·49
Eserine SO_4 ..	0·39	0·35–0·43
Neostigmine $MeSO_4$	0·27	0·25–0·30

DISCUSSION

This series of compounds presents certain analogies and physical similarities to the polymethylene bistrimethyl ammonium compounds of the general formula

$$\bar{I}Me_3\overset{+}{N}(CH_2)n\overset{+}{N}Me_3\bar{I}$$

examined by Willstätter and Heubner (1907), Barlow and Ing (1948), and Paton and Zaimis (1949). These last authors showed that activity as paralysing agents was greatest when $n=9$ to 12, and that all compounds where $n>7$ showed some activity as anticholinesterases, the dodecyl member being the most potent against the true enzyme. In our group of specific cholinesterase inhibitors the distance between the quaternary nitrogens is about equivalent to that in the 1:12 dodecyl bistrimethylammonium ion. Despite the chemical analogies between the two series, the anticholinesterase activity of our compounds is much more prominent than the paralysing effect. Another series, of the general form



and thus intermediate to decamethonium and those compounds described here, was tested by Randall (1952). Paralysing activity was again uppermost. However, the members of another closely related series, examined by Funke and Depierre (1950), and mentioned later, were predominately anticurare agents.

Neuromuscular Block.—The neuromuscular blocking action of anticholinesterases has been described frequently (Briscoe, 1936; Rosenblueth, Lindsley, and Morison, 1936; Lehmann, 1946; Krop, 1947) and has usually been ascribed to an accumulation of undestroyed acetylcholine. At first this was thought to be a likely mode of action of our series, but cholinesterase inhibition and paralysing activities are not simply related. It seems that the double quaternary nature of the molecule is not essential, for compounds with only one nitrogen (e.g., 153C47, 26C48, 101C48) are as potent paralysing agents as those with two, but are weaker inhibitors of " true " cholinesterase. The paralysis was due to an action at the myoneural junction, since the muscle response to direct stimulation was unaffected, and conduction along the phrenic nerve was not blocked by concentrations of 62C47 which reduced the diaphragmatic twitch.

The block from our compounds resembles that of (+)-tubocurarine in some respects, as, for example, in failure of the diaphragm to maintain a tetanus and in the action being reversed after tetanic stimulation; but it is unlike (+)-tubocurarine in not being antagonized by neostigmine. However, these paralysing compounds are themselves anticholinesterases.

Barnes and Duff (1953) have recently shown that inhibition of cholinesterase *per se* does not block neuromuscular transmission. Our evidence, too, suggests, that the paralysing action of these cholinesterase inhibitors is independent of the inhibition of the enzyme—whereas, in a series of compounds with a polymethylene chain uniting two *iso*quinoline groups, curariform activity paralleled inhibition of the true enzyme (Smith, Pelikan, Maramba, and Unna, 1953).

Curare Antagonism.—The anticurare action resembles that of neostigmine more than the direct stimulating effect of 3-hydroxyphenyltrimethylammonium iodide, and of its mono-ethyl derivative (Randall, 1950), or of catechol (Mogey and Young, 1949).

Most of our compounds with only one quaternary nitrogen were very weak antagonists. It may be that two such nitrogens are required for maximal anticurare activity.

This curare antagonism may not be simple. It may be due (*a*) to anticholinesterase effects, or (*b*) to a direct stimulant action at the myoneural junction, or (*c*) to a combination of these, or (*d*) to some other effect. (*a*) Significant correlation between anticurare and anticholinesterase actions was found by Blaschko, Bülbring, and Chou (1949) in some neostigmine analogues, and also by Hobbiger (1950) for tetraethylpyrophosphate, physostigmine, neostigmine, and two homologues. On the other hand, Aeschlimann and Stempel (1946) and Randall and Lehmann (1950) found no such correlation in closely related series. Correlation between anticurare and anticholinesterase actions, although positive, was not statistically significant in our series. (*b*) Compound 62C47 and an unsaturated derivative not so far mentioned (729C47,

had a nicotine stimulating type of action on the frog rectus, whereas 25C48, 297C50, and 298C50 had not. These last three, although devoid of stimulating action on frog rectus, were among the most potent as anticurare agents and cholinesterase inhibitors. Randall and Lehmann (1950) found that the direct stimulating action on denervated muscle of phenyl-trimethylammonium bromide and analogues did not correlate with anticurare activity, but that these two actions were closely related in muscle stimulated through its nerve.

A series of compounds examined by Funke and Depierre (1950), very similar in structure to our series, had the general formula

These were found to be anticurare agents, and when n=3, anticholinesterase activity and curare antagonism were very marked. They did not say whether their compounds showed any selectivity for true cholinesterase, although they did report that they inhibited the red cell esterase which, in most species, is predominantly true cholinesterase. As with our compounds, but unlike " Tensilon " (3-hydroxyl-phenyl dimethyl-ethyl ammonium iodide), none of their series antagonized a massive dose of (+)-tubocurarine.

The finding that atropine did not reduce the inhibition of cholinesterase *in vitro* by these compounds is in agreement with Frey's results (1948) for physostigmine, although high concentrations of atropine prevented the anticurare action, as with neostigmine. This is of interest in view of the synergism reported by McDowall (1949) between atropine and (+)-tubocurarine. Atropine blocked some of the actions of these anticholinesterases (e.g., salivation and miosis in the mouse and rabbit), although it did not reduce the toxicity. Kimura and Unna (1950) showed that 2.0 mg./kg. atropine sulphate intravenously more than doubled the LD50 of physostigmine.

Cholinesterase Inhibition.—The outstanding characteristic of the series is cholinesterase inhibition, several members having the same order of activity as eserine and neostigmine on true cholinesterase when acetylcholine is used as substrate. Some—e.g., 25C48, 297C50, and 298C50—show little or no inhibition of pseudo-cholinesterase, although they are potent inhibitors of true cholinesterase. In fact, the characteristic most common to this group of anticholinesterases is their low activity against pseudo-cholinesterase. The activity against true cholinesterase varies within very much wider limits, thus causing the degree of specificity to fluctuate from ten-fold or so to about one hundred thousand-fold.

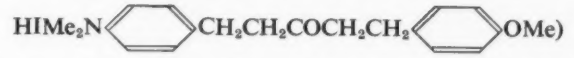
The most specifically effective are compounds 62C47, 25C48, 297C50, 298C50, and 455C50. These are all symmetrical molecules, and the distance between the two quaternary nitrogens is of the order of that obtaining in 1 : 12 dodecyl-bis-trimethylammonium. The less active compound 142C48 is also symmetrical and has a similar inter-nitrogen length ; but the centre around which it is symmetrical is not CO but $\begin{matrix}\\ \\ \end{matrix}\!\!>\!\!\text{HCOCOPh}$. Even when the central group is $\begin{matrix}\\ \\ \end{matrix}\!\!>\!\!\text{CO}$, attachment of a radical larger than propyl to the quaternary nitrogens lowers the specificity. Though some specificity persists in those compounds with nearly the same total length of central chain, but with a single quaternary nitrogen, it is much less than in the five compounds mentioned above, and the activity against both enzymes is of a lower order.

The selective reversible inhibition of the di-allyl compound, 297C50, was described under the number 284C51 by Austin and Berry (1953), and that of 62C47 by Burgen (1949b) and Todrick (1954).

The selectivity shown for true cholinesterase is much greater than that described by Adams and Thompson (1948), who found $\beta\beta'$-dichloro-diethyl-N-methylamine (DDM)—the first example, apart from caffeine (Zeller and Bissegger, 1943), of a selective inhibitor of true cholinesterase—to be 10 to 30 times as active on pigeon brain esterase as on human plasma enzyme. The selectivity is greater than that found by Paton and Zaimis for the polymethylene bis-trimethylammonium series, and by Hawkins and Mendel (1949) for the neostigmine analogue, Nu 1250.

Equally selective inhibitors of pseudo-cholinesterase are now known. There are, for example, *iso*OMPA—which, according to Aldridge (1952), is 10,000 times more active against pseudo- than true cholinesterase—and mipafox, which Davison (1953) has reported to be even more selective for pseudo-cholinesterase (on chicken enzymes). It should be noted, however, that these inhibitors of pseudo-cholinesterase have a practically irreversible action, whereas the effects of those inhibitors of true cholinesterase described in this paper are reversible.

It is possible that the action of these compounds on cholinesterase is due to the two quaternary ammonium groups (cf. Sanz, 1945), since compound 83C48 (not in Table I : structure



gave a *p*I 50 of 3.0 for true cholinesterase and 2.0 for pseudo-cholinesterase ; and since compounds 153C47, 26C48, and 101C48, with but one quaternary nitrogen each, are among the weakest of our series. Nevertheless, the bisquaternary compound

is not an anticholinesterase. However, increasing the distance between the benzene rings by the insertion of four more methylene groups, so giving 71C48, decreases its paralysing effect and introduces anticholinesterase activity (Glock, Mogey, and Trevan, 1948).

It has been suggested (Bergmann, Wilson, and Nachmansohn, 1950a) that the surface of true or acetylcholinesterase has two active centres, one an anionic site bearing a unit negative charge which combines with the cationic head of the substrate or inhibitor, and the other an esteratic site which has two functioning groups. One of the esteratic groups combines with the electophilic carbon of the ester carbonyl of acetylcholine, and the other with one of the two oxygens. The flexibility of the central chain of some of our compounds might allow them to become attached to some of such centres tailored to the measurements of acetylcholine. It is, however, surprising that 729C47 should also fit equally well, for its spine is somewhat more rigid : the double bonds here will, of course, introduce electron fluidity. The absence of a ketone group in the central chain is associated with a decrease in the selective affinity for true cholinesterase, although 71C48 still shows the typical effects. A greater drop of activity results from the removal of one of the quaternary nitrogens.

These observations support the later suggestion of Bergmann, Wilson, and Nachmansohn (1950b) that the true cholinesterase molecule has two anionic sites as well as the esteratic focus. The distance between these sites may be spanned by

the inhibitor. The Coulomb attraction between the onium groups of the compound and the anionic centres of the enzyme is probably the important factor in retaining the inhibitor in position. Van der Waals' forces will add to this attraction : these will increase, with the addition of radicals larger than methyl on the quaternary nitrogen, until a size is reached which hinders attachment because of its bulk. One butyl group appears sufficient to do this. But if this applies to the active site of cholinesterase it is difficult to see why it does not also apply to the point of action of acetylcholine at the neuromuscular junction.

The results show that our most selective compounds have activities of the orders of those of neostigmine and eserine on true cholinesterase. With some substrates (e.g., acetyl-β-methyl choline) they were less active. Burgen (1949b) found that 62C47 was less active than eserine *in vitro*, but he was concerned only with acetyl-β-methylcholine as substrate (private communication). We can confirm this. He also found it (1949a) to be more active than eserine in potentiating the red tear response in rats.

Tests for correlation between the various types of action show that paralysing activity bears no relationship to the others. There is a significant correlation between toxicity and anticholinesterase activity. The positive correlation that exists between anticurare and anticholinesterase activities is fairly good, although not statistically significant —due, possibly, to the small number of observations, or perhaps because this action may depend on more than one effect.

It is difficult to see new therapeutic applications for these compounds, but they will serve a useful purpose as heuristic tools—especially if used in conjunction with selective inhibitors of pseudocholinesterase.

Summary

1. A series of compounds, whose chief chemical characteristic is an arrangement of two quaternary nitrogens joined by a spine, which varies in composition but not much in length, has been examined for various pharmacodynamic effects.

2. The outstanding effect is selective reversible inhibition of true cholinesterase.

3. Some of the compounds are as active as neostigmine or eserine.

4. Some are the most selective inhibitors of true cholinesterase so far described.

We are glad to acknowledge the co-operation of Drs. J. R. Catch and F. C. Copp, who synthesized these compounds. We wish to thank Mrs. A. Turnbull, Miss H. Parsons, and Mr. F. Huggins for technical assistance, and Mr. P. A. Young for statistical calculations. Dr. A. C. White rendered philosophical advice and valuable criticism.

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THE PART PLAYED BY INHIBITION OF CHOLINESTERASES OF THE CNS IN PRODUCING PARALYSIS IN CHICKENS

BY

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Bidstrup and Hunter (1952) reported two cases of poisoning with *NN'*-di*iso*propylphosphorodiamidic fluoride (mipafox) which exhibited an unexpected symptomatic picture. Three people developed acute poisoning while engaged on a pilot plant manufacturing this substance. Recovery from the acute phase of the illness followed the administration of atropine in large doses, but in the three weeks after the onset of symptoms two of the patients developed paralysis of the limbs similar to that which follows triorthocresol phosphate (TOCP) poisoning. Smith and Lillie (1931) showed that this was accompanied by a demyelination of the nerve sheath. Petry (1951) has described similar effects after poisoning with *OO*-diethyl *O-p*-nitrophenyl phosphorothioate (parathion). Paralysis in man following intoxication with TOCP has been described frequently. Thus Lorot (1899) reported neuritis in tuberculous patients after treatment with "phosphocreosote." During prohibition in the United States, TOCP was used as an adulterant in "Ginger Jake," a drink then popular. As a result, several thousand cases of paralysis occurred (Smith, Elvove, and Frazier, 1930). Ter Braak (1931) and Sampson (1938) reported several others from the continent of Europe. In 1944, Hunter, Perry, and Evans described further cases in industries using TOCP as a "plasticizer."

The appearance of these effects after poisoning with the new organophosphorus insecticides has resulted in researches designed to determine the mechanism by which the paralysis is caused.

Three compounds—TOCP, DFP, and mipafox—will produce paralysis in chickens (Barnes and Denz, 1953; Earl and Thompson, 1952; and David, 1951). Koelle, Gilman, and Binzer (1946) administered DFP over a long period to cats and dogs with similar results. Bloch (1941), and Hottinger and Bloch (1943), had attempted to explain such effects in terms of the anticholinesterase properties of TOCP. They suggested that the inhibition of cholinesterase resulted in excessive concentrations of acetylcholine at the nerve endings and that this caused the paralysis.

More recently the effects of TOCP upon a number of widely different enzyme systems have been studied by Earl, Thompson, and Webster (1953). They examined glucose and pyruvate oxidation by brain, brain amine oxidase, brain cephalinase, pancreatic lecithinase, and trypsin. All these systems were insensitive to this compound. The tributyrinase activity of the spinal cord of hens poisoned with TOCP was moderately reduced. In every experiment, however, the pseudo-cholinesterase of the spinal cord was inhibited to a greater extent than was either the true cholinesterase of the tissue or the tributyrinase.

Both DFP and mipafox, in company with TOCP, are selective inhibitors of pseudo-cholinesterase. Thus all the available evidence suggested that paralysis might well be a consequence of inhibition of pseudo-cholinesterase. Earl and Thompson (1952), in discussing these results, suggested that paralysis may result from the inhibition and continued depression of the pseudo-cholinesterase of the spinal cord. Since these authors had at that time worked with only TOCP, it was desirable to examine this hypothesis further. We have, therefore, examined the possible paralysing effects of five powerful anticholinesterases: di*iso*propyl phosphorofluoridate (DFP), *iso*propyl methylphosphonofluoridate (sarin), ethyl *NN'*-dimethylphosphoroamidocyanidate (tabun), 3:3-dimethyl-*n*-butyl 2-methylphosphonofluoridate (soman), and *iso*propyl-ethylphosphonofluoridate (ethyl sarin).

We set out to answer two questions:

1. Would all five substances produce paralysis in chickens?

2. Could the cholinesterase changes be related to the possible ability of these substances to cause paralysis?

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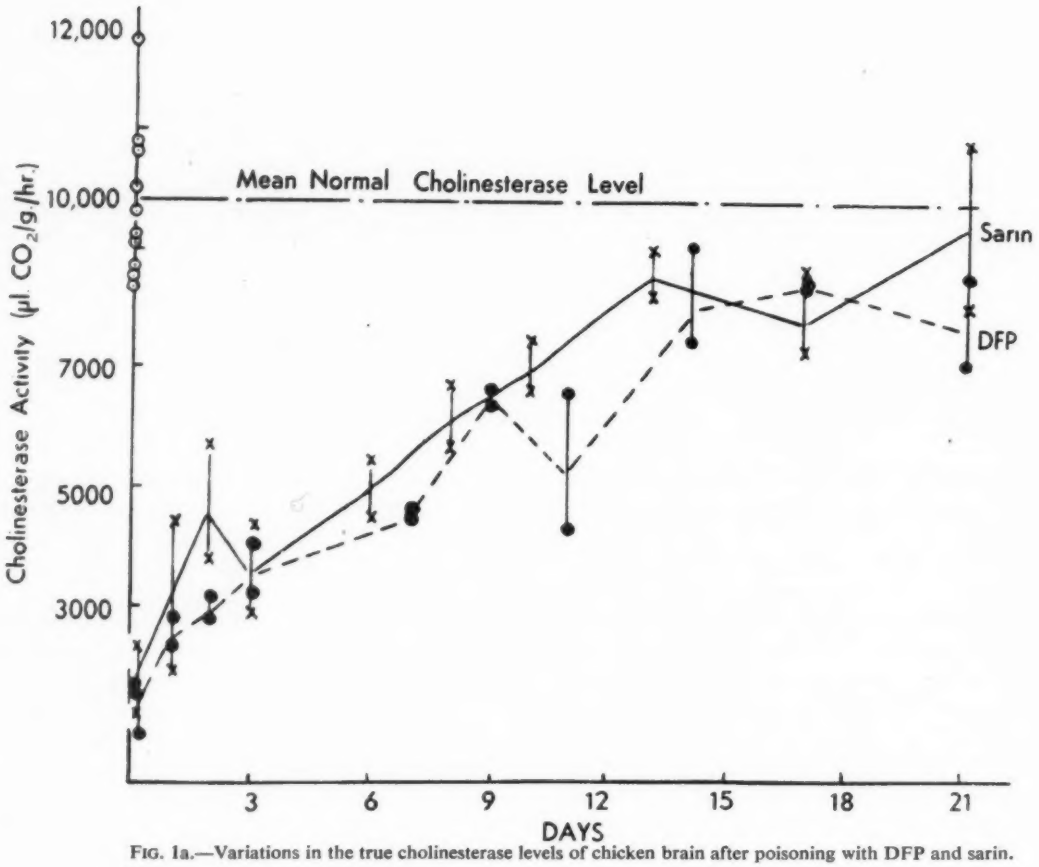

FIG. 1a.—Variations in the true cholinesterase levels of chicken brain after poisoning with DFP and sarin.

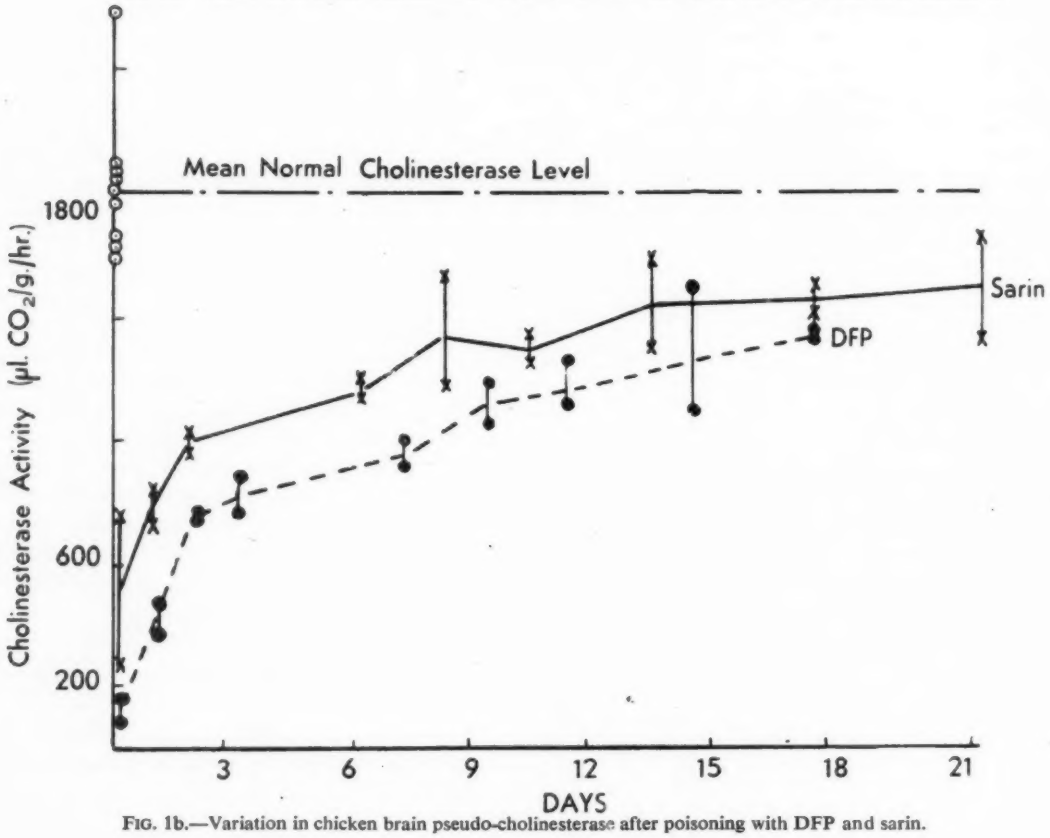

FIG. 1b.—Variation in chicken brain pseudo-cholinesterase after poisoning with DFP and sarin.

METHODS

Chickens were used, since several workers have described techniques for the production of paralysis in these with TOCP and DFP (Smith, Elvove, and Frazier, 1930; Smith and Lillie, 1931; Smith, Engel, and Stohlman, 1932; Earl and Thompson, 1952; and Barnes and Denz, 1953). Birds of mixed breeds weighing 1,800–3,500 g. were used.

The organophosphorus compounds were given subcutaneously in a 10^{-3} dilution in physiological saline. DFP, however, was used in a 1% w/v solution, in physiological saline containing 10% ethyl alcohol to aid solution.

The doses used were within the lethal range, so atropine sulphate (1 mg./kg.) was given subcutaneously immediately after each dose of the toxic substance.

Enzyme determinations were made upon the brain, spinal cord, and blood. After removal the brain and spinal cord were quickly homogenized in 0.25 M-sodium bicarbonate. For assay of the *true* enzyme of the brain 1 g. of tissue was homogenized with 150 ml. of the bicarbonate solution; for the *pseudo*-cholinesterase of the brain, and for both *true* and *pseudo*-cholinesterases of the spinal cord, 1 g. was homogenized with 25 ml. Blood was obtained from a wing vein and collected into dry heparin, the plasma separated off and diluted with 11.5 volumes of bicarbonate solution.

The activity of the enzymes was determined in the Warburg apparatus using acetyl-β-methylcholine chloride (0.03M) for the true enzyme and butyrylcholine iodide (0.03M) for the pseudo enzyme. The results are expressed as $\mu l.CO_2$ produced in 1 hr. by 1 g. tissue (or 1 ml. plasma).

RESULTS

Doses within the lethal range were used and, despite the administration of atropine, some deaths occurred.

With DFP, paralysis was produced regularly; immediately after dosing each bird exhibited signs of ACh poisoning which persisted for 24–48 hr. They recovered from this; but 10 to 14 days after dosing they showed signs of progressive weakness of the legs. The subsequent picture was identical with that described by Earl and Thompson (1952) after TOCP poisoning, and by Barnes and Denz (1953) after treatment with some insecticides. After the onset of paralysis the general health of the birds deteriorated. They lost weight rapidly and, about a week later, many showed cyanosis of the combs and gaping of the beak. At this stage many died. Histological examination showed evidence of demyelination of the ascending and descending tracts of the spinal cord.

In no chicken was there any sign of paralysis with sarin, tabun, soman, or ethyl sarin, although both large and small doses were repeated for several weeks. Sarin, for example, was administered twice a week for five weeks. Despite this long period there was no evidence of paralysis, although a number of the birds were observed for as long as three months. This was in marked contrast to DFP. The doses used were such that many of the birds died after two or three weekly injections.

Changes in the Cholinesterase Levels of Tissues and Plasma in Relation to the Onset of Paralysis

As indicated in the introduction, Earl and Thompson (1952) have suggested that paralysis might result from prolonged inhibition of pseudocholinesterase. Because of our failure to produce paralysis with the agents described, it was important to compare the changes in the true and pseudocholinesterases of the brain, spinal cord, and plasma in groups of chickens injected with DFP and sarin.

Normal levels for both the true and the pseudoenzymes of the brain and spinal cord were obtained by examining the tissues of a group of 10 normal birds. Mean values, together with the standard deviations of the individual values, are shown in Table I.

TABLE I

NORMAL LEVELS OF TRUE AND OF PSEUDO-CHOLINESTERASE IN CHICKEN BRAIN AND SPINAL CORD

(Mean values from 10 birds. The substrates were acetyl-β-methylcholine (0·03M) and butyrylcholine (0·03M). Cholinesterase activity is expressed as $\mu l.CO_2/g./hr.$)

	Brain		Spinal Cord	
	True	Pseudo	True	Pseudo
Mean ..	9,828	1,810	1,689	1,247
Range ..	8,400–12,600	1,600–2,400	1,360–2,280	1,020–1,520
Standard deviation	1,260	229	393	153

Ten chickens were injected with DFP and 10 with sarin. Each bird received atropine sulphate. They were then sacrificed in pairs, one pair from each group, the first pair being killed 2 hr. after dosing. The levels of enzyme activity at this time are shown in Table II. Further pairs were killed at varying times over a period of three weeks, as illustrated in Fig. 1a and Fig. 1b, which show the variations in enzyme levels.

Brain.—The true cholinesterase of the brain was depressed equally by the two agents to approximately 20% of the mean normal value.

The rate of recovery was similar in each group (Figs. 1a and 1b). The level reached 50% of

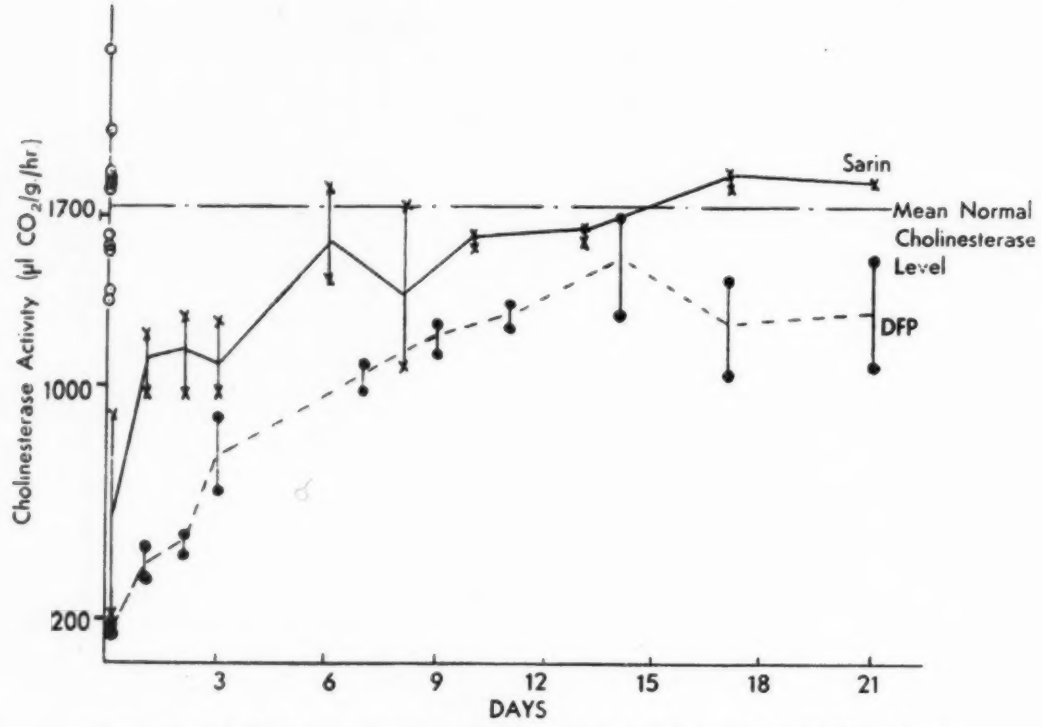

FIG. 2a.—The variation in true cholinesterase of chicken spinal cord after poisoning with DFP and sarin.

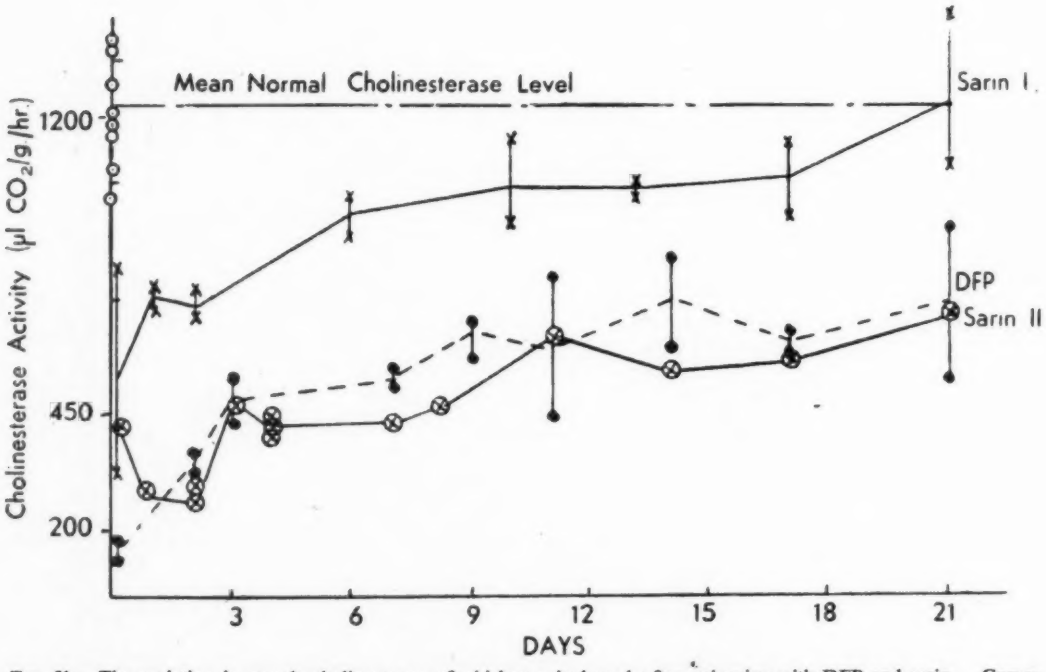

FIG. 2b.—The variation in pseudo-cholinesterase of chicken spinal cord after poisoning with DFP and sarin. Curves Sarin I and DFP show the rate of reappearance of enzyme after a single dose of each agent. Curve Sarin II shows corresponding changes after repeated dosing with smaller doses of sarin, to reduce cholinesterase to level comparable with that obtained with DFP.

TABLE II

CHOLINESTERASE ACTIVITY (% OF NORMAL) IN CHICKENS TWO HOURS AFTER POISONING WITH DFP AND SARIN

(Each estimate is the mean of observations upon four birds)

Tissue	True		Pseudo	
	DFP	Sarin	DFP	Sarin
Plasma ..	—	—	0	19
Brain ..	12·5	17·5	6	28
Spinal cord	6	33·5	10	47

normal in approximately a week and was back to within the lower normal limits in two to three weeks. The pseudo-cholinesterase of the brain was inhibited rather more strongly by DFP than by sarin.

The pseudo-cholinesterase of the DFP-treated birds was reduced to 5% or normal, whereas with sarin 25% activity remained. Rates of recovery were again similar. There was a rapid initial rise to about the 50% level, then the recovery rate slowed down, and even at the end of three weeks the enzyme level was not back within the normal range.

Spinal Cord.—Neither the true nor the pseudo-cholinesterases of the spinal cord was inhibited by sarin to the same extent as with DFP (Fig. 2). The true esterase was inhibited by sarin to only 30%, whereas with DFP it was reduced to 8%. The pseudo enzyme was reduced to 40% by sarin and to 10% by DFP. Again, the rates of recovery were essentially the same as for the true enzyme, the levels returning to within normal limits in approximately a fortnight after both agents. The level of the pseudo-cholinesterase in the DFP-treated birds was, however, substantially below normal for the whole three weeks of the experiment; this was in marked contrast to the sarin-treated birds, in which the enzyme reached normal limits in approximately a week after dosing. In view of the suggestions of Earl and Thompson (1952), this observation was felt to be significant. It was therefore decided to carry out another experiment in which the pseudo esterase of the spinal cord would be reduced to less than 20% and kept at a level comparable with that found in the DFP-treated birds of the previous experiment.

The essential problem was the administration of sufficient sarin to depress the enzyme to less than 20% of normal, to maintain it at or near this level for a prolonged period, and still keep the bird alive. If sarin is given in a series of appropriately spaced small doses, the animal can tolerate considerably more than if it were given in a single large dose (Berry and Davies, 1951). By giving sarin in this way the necessary degree of inhibition of the pseudo-cholinesterase of the spinal cord was obtained.

Birds were killed at intervals for the assay of the tissue enzyme levels. The remainder were then kept under observation for a further three weeks. The changes in the spinal cord pseudo-cholinesterase are shown in Fig. 2, from which it can be seen that the enzyme level in this group of birds was: (i) reduced to a level comparable with that which obtained in the DFP group of the previous experiment, and (ii) was maintained at a low level

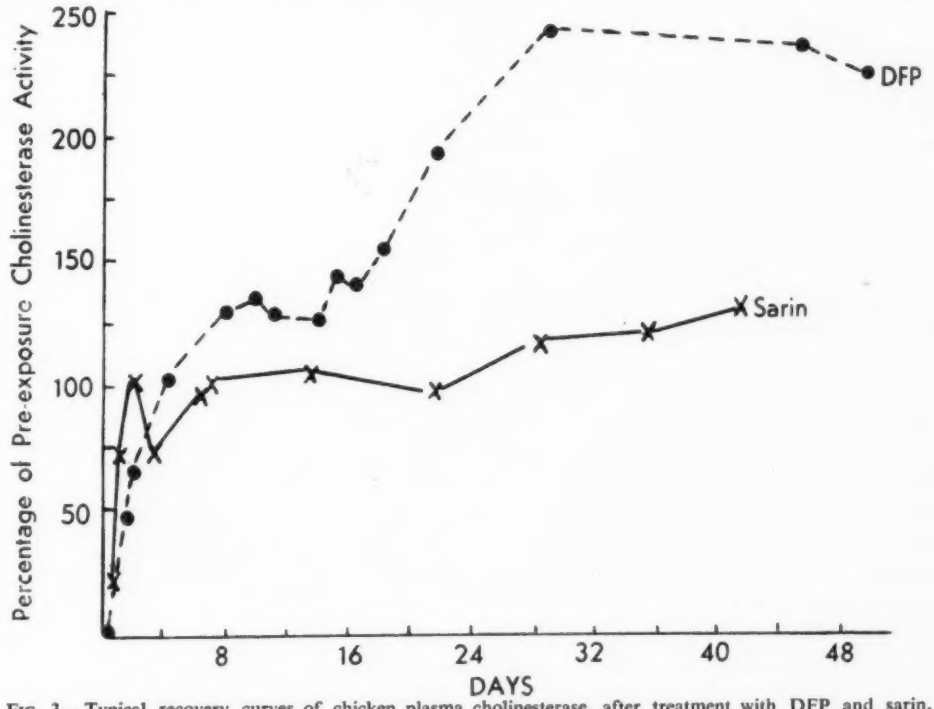

FIG. 3.—Typical recovery curves of chicken plasma cholinesterase, after treatment with DFP and sarin. Note the early recovery and the subsequent slow and prolonged rise above normal after DFP. A similar but much less marked rise occurs after sarin.

for an equally long time. There were no signs of paralysis in this group.

Variations in the Level of Plasma Cholinesterase in Fowls After Dosing

Observations were also made upon variations in the levels of plasma esterase. With the doses used, the enzyme was substantially inhibited. With DFP all the enzyme was destroyed, but with sarin there was only 85% inhibition.

The rate of return of the plasma esterase to pre-exposure levels was rapid with all the agents. Complete recovery took only 2–5 days, but the plasma activity increased substantially beyond normal in the succeeding three weeks, and remained at this level for a further 30 days (Fig. 3). Thus, with DFP, there was a mean increase of 150% above the normal (5 birds); with the other agents there was a 20–50% increase. The possibility that this increased activity, since it was measured against butyrylcholine, was due to another type of cholinesterase—a butyrylcholinesterase which did not hydrolyse acetylcholine—has been considered. This type of phenomenon has been noticed by Ellis (1947), who showed that guinea-pig kidney contains a benzoylcholinesterase which does not hydrolyse acetylcholine. He showed that this enzyme also occurs in some, but not all, rabbits, and that even in one rabbit its occurrence varies from time to time (Ellis, 1947).

The nature of the plasma cholinesterase was therefore investigated both before and one week after dosing. At the latter time it was significantly above the pre-exposure value. Substrate specificity patterns against acetylcholine, acetyl-β-methylcholine, benzoylcholine, butyrylcholine, and propionylcholine were determined. There were no differences. The behaviour of selective inhibitors was also examined. Austin and Berry (1953) described two inhibitors which were highly selective towards each enzyme: 1:5 bis (4 allyldimethylammonium phenyl) pentan-3-one dibromide (284C51, Wellcome), a reversible inhibitor highly selective for the true enzyme, and *NN'*di*iso*propylphosphorodiamidic anhydride (DPDA), an irreversible inhibitor which is highly selective for the pseudo enzyme. Tests with these two inhibitors were carried out on the plasma before and after exposure. No differences were seen. Thus it is highly probable that this increased activity is due to an increase in concentration of the normal plasma enzyme.

Discussion

The purpose of this investigation has been twofold. Firstly, to find whether all anticholinesterases cause paralysis under laboratory conditions; and secondly, to observe the changes in the true and the pseudo-cholinesterases of brain, spinal cord, and blood in chickens. The results have been clear-cut. Chickens were used because they had been shown to be particularly susceptible to the paralysing action of TOCP. Furthermore, Earl and Thompson (1952), Barnes and Denz (1953) and David (1951) had already shown with DFP, TOCP, and mipafox that paralysis and demyelination could be produced relatively easily in this species. We have confirmed this repeatedly with DFP. We have, however, been unable to produce paralysis with tabun, sarin, soman, or ethyl sarin, not only with large single doses but also with repeated large doses at weekly and twice weekly intervals over periods varying from two to nine weeks. No other effects which could be interpreted as resulting from demyelination developed. This aspect of the investigation may thus be summarized as follows: in a particularly susceptible species, it has not been possible to produce paralysis with any of the substances examined except DFP.

Davison (1953) has come to the same conclusion. He studied a number of organophosphorus compounds which were all selective inhibitors of pseudo-cholinesterase; recovery from the effects was rapid—and had a similar pattern—whether paralysis was produced or not.

Reference has been made in the introduction to the ideas of Bloch (1941), Hottenger and Bloch (1943), and Earl and Thompson (1952) upon the possible significance of cholinesterase inhibition in relation to the aetiology of demyelination. Our results indicate that the inhibition of neither true nor pseudo-cholinesterase of chicken brain or spinal cord can be the direct causative factor, since with only one—DFP—of five powerful anticholinesterases was it possible to produce paralysis. In a carefully designed experiment, illustrated in Fig. 2, similar enzyme changes were brought about by DFP and sarin; although paralysis developed in the DFP-treated birds, there was none in the sarin group. Thus, whatever the cause of paralysis, it is unlikely that the inhibition and continued depression of the pseudo-cholinesterase of the brain or spinal cord plays a direct part in its development.

The plasma enzyme changes are interesting. The esterase activity returns to pre-exposure levels in 2–5 days. This is in marked contrast to the rate of recovery after TOCP in chickens, and mipafox in man. With TOCP it took at least three weeks to return to within normal limits (Earl and Thompson, 1952). In poisoning with mipafox in man the return to normal levels was significantly delayed (Callaway, Davies, and Risley, 1952).

An interesting observation is the post-intoxication increase of plasma esterase to levels very significantly higher than normal. This was particularly marked following DFP. Koelle and Gilman (1946) and Locker and Siedek (1952) have reported a significant increase in blood and tissue cholinesterase over pre-exposure levels after dosing with anticholinesterases. Koelle and Gilman also noted this in rats, but did not comment on it. Locker and Siedek, however, have made a separate study of the phenomenon, following the administration to guinea-pigs and rats of *NNN'N'*-tetramethylphosphorodiamidic anhydride (Pestox III), *NNN'N'*-tetramethylphosphorodiamidic fluoride (Pestox XIV), parathion, and mipafox. They found a rapid increase of 40–60% in the cholinesterase activity of plasma, erythrocytes, liver, and brain. The increase was more marked with small doses and occurred comparatively rapidly—in 1–3 days after dosing. They suggested that these effects might be due to a different distribution of the alkylphosphates in the body and to a stimulatory effect of low concentrations upon the formation or activity of the enzymes.

The observations here reported with chickens appear to differ from those in the literature in at least three respects. Firstly, the rise after DFP is very much greater than that reported by Locker and Siedek. Secondly, in guinea-pigs intoxicated with insecticides, the increase occurred within 1–3 days. In our experiments the rise was progressive up to three weeks, and the higher level of plasma cholinesterase was maintained in some birds for at least another month. Thirdly, Locker and Siedek suggest that the effect is due to a stimulatory effect of low concentrations of the anticholinesterases. We used only one dose level of DFP, but this was well within the lethal range. The response to other phosphorus compounds was much less than that to DFP. Thus it may be that this stimulation of esterase production or activity is dependent upon the agent initially used to inhibit the enzyme.

It is interesting to recall in this connexion that Hollinger, Rossiter, and Upmalis (1952), and Hollinger and Rossiter (1952), have found considerable increases in acid-phosphomonoesterases, 5-nucleotidase, and glucuronidase in the cat's sciatic nerve after nerve section. These changes reached their peak in the same time that maximal effects were observed in our experiments with cholinesterase, and over the same interval that it took for the toxic effects to develop.

Summary

1. Five potent anticholinesterases, DFP, sarin, tabun, soman, and ethyl sarin, have been examined for their chronic effects in chickens.

2. Paralysis was produced by DFP only.

3. It is improbable that paralysis following DFP is due directly to the inhibition and prolonged depression of either the true or the pseudocholinesterase of CNS or blood.

4. The plasma cholinesterase was markedly inhibited after poisoning with each of the compounds. The rate of return to normal levels was always rapid (3–5 days). The cholinesterase level of the plasma continued to rise to levels substantially higher than normal; this was particularly striking with DFP.

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ACETYLCHOLINE PRODUCTION IN ANIMALS POISONED BY DIETHYL-*p*-NITROPHENYL PHOSPHATE (PARAOXON)

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During a study of the toxic action of diethyl-*p*-nitrophenyl phosphate (paraoxon, E600), which is a powerful inhibitor of cholinesterase (Aldridge, 1950), it was found that anaesthetized fully atropinized rabbits could be revived from an otherwise lethal dose by artificial respiration, and that they then became insensitive to further injections of the same dose of this inhibitor (Barnes, 1953). It was noted that the animals given paraoxon remained very sensitive to the effects of ACh and that this sensitivity increased temporarily after each dose of inhibitor. If death from failure of respiration after the first dose of the inhibitor could be attributed to the action of excessive amounts of ACh in the brain or at other important sites, the insensitivity of the anaesthetized rabbit to subsequent doses might result from a reduced ACh production in the poisoned animal.

Douglas and Paton (1951), using an inhibitor with very similar properties (TEPP, tetra-ethyl-pyrophosphate), found that levels of 40 mμg. ACh/ml. were reached in the blood of the cat after a large single dose. They stated that much of this ACh came from the bowel. Stewart (1952), using the isolated *Venus mercenaria* heart, assayed the blood of rats dying from paraoxon poisoning and found it to contain 8–30 mμg. ACh/ml. ACh was "just detectable" in the blood of monkeys at the time respiration failed after poisoning with di*iso*-propyl phosphofluoridate (DFP, dyflos). More recently, Perry (1953) has amplified the work of Brown and Feldberg (1936), who found that ACh production from the perfused eserinized cervical sympathetic ganglion of the cat fell rapidly during periods of continued stimulation. Perry found that this fall in output did not occur in the absence of eserine. This suggested that a prolonged inhibition of cholinesterase might be associated with a diminished production of ACh.

The experiments to be described were designed to test these hypotheses.

METHODS

Male rabbits (1.5–3 kg.) were anaesthetized with urethane (1 g./kg.) given intravenously as a 25% solution. Male and female dogs (9.5–16 kg.) and cats (2.5–3.5 kg.) were anaesthetized with pentobarbitone sodium (30 mg./kg., i.v.).

In all animals a tracheal cannula was inserted and the carotid blood pressure was recorded from a Hg manometer. A record of the respiratory movements was obtained by a thread tied to the lower part of the chest. To obtain blood for ACh assay, a polythene cannula was introduced into the superior (rabbits) or inferior (dogs and cats) vena cava from a peripheral vein.

Blood samples (7–10 ml.) were withdrawn into 0.2 ml. of 1% heparin, and eserine (10^{-5} M) was added immediately after. The assays were carried out within a few minutes.

All drugs were given intravenously. Atropine was given freely except in experiments where the dose was deliberately kept as low as possible. It was given before the first injection of paraoxon and with each dose; it was also given between doses if the condition of the animal suggested that it might be beneficial. The total dose administered ranged from 2–7 mg./kg. Paraoxon was prepared as a solution in alcohol and injected as a 1:10 emulsion made by diluting this in atropine solution immediately before injection.

The isolated rat diaphragm preparation was essentially that described by Bülbring (1946), with the small bath (7 ml.) suggested by Burgen, Dickens and Zatman (1949). The diaphragm was stimulated by supramaximal rectangular impulses of 0.2 msec. duration at a frequency of 50/sec. for 20 min., and the contents of the bath then immediately removed. When the assays were not done at once, this fluid was acidified with HCl to *p*H 4, frozen at −15° C., and kept so until just before assay. Eserine (5×10^{-5} M) or paraoxon (4×10^{-6} M) was added to the bath at least 5 min. before stimulation was started.

ACh in the blood was assayed on the leech dorsal muscle. Normal blood had little or no effect on this preparation. At least 7 ml. blood was required for each assay; this limited the number of samples that

could be taken from a single animal. The ACh levels were expressed as millimicrograms (mμg.) base per ml. blood. Fluid from the diaphragm bath was assayed on the blood pressure of the chloralosed cat. The techniques and precautions described by MacIntosh and Perry (1950) were followed.

Results

Rabbits.—Rabbits were given successive doses of 0.5 mg./kg. paraoxon at intervals of 30 min. and their response was similar to that already described (Barnes, 1953); the animals died with progressive respiratory and cardiac failure after three or four doses. The ACh level in the blood was determined 10–15 min. after each dose of paraoxon; the levels (mμg./ml. whole blood) in five animals are given in Table I.

TABLE I

BLOOD LEVELS OF ACh (MμG. BASE/ML. WHOLE BLOOD) IN ATROPINIZED RABBITS 15 MIN. AFTER SUCCESSIVE DOSES OF PARAOXON 0·5 MG./KG.

Interval between doses 40–45 min.

Dose Sequence	Rabbit No.				
	1	2	3	4	5
1st dose	4·6	26	12	9	16
2nd „	18·6	35	—	16	—
3rd „	28·0	44	16	30	26
4th „	28·0	—	34	43	—

There was always a progressive rise in the blood ACh. In one rabbit the level was followed for 90 min. before giving a further dose of paraoxon; in this period it fell to about 50% of the level attained just after the inhibitor had been given.

Dogs.—The experiments were continued on dogs because they could provide a greater number of blood samples for assay. In four animals the initial dose of paraoxon was 3 mg./kg. and this caused temporary respiratory failure which was relieved by a short period (2–3 min.) of artificial respiration. One dog was given a larger initial dose (10 mg./kg.), but it died in spite of artificial respiration. The animals that survived the initial dose of paraoxon were quite unaffected by subsequent doses of up to 20 mg./kg., and no dog died of the effects of paraoxon alone, though some were observed for as long as 7 hr., and received total doses of 40–70 mg./kg. They remained sensitive to ACh, and an intravenous injection of 500 μg. would arrest respiration and kill them.

The blood levels in four dogs 30 min. after receiving successive doses of paraoxon are given in Table II. The blood ACh levels rose after the first and second doses of paraoxon, but thereafter there was little further change. In one animal (Dog 3, Table II) the blood level was followed for 4 hr. after the second dose of paraoxon; it fell from 20 mμg./ml. to 15 mμg./ml. at 3 hr., and to 10 mμg./ml. at 4 hr. A third dose of paraoxon then raised it promptly to 20 mμg./ml. The rate of fall of the blood ACh is so slow that slight variation in the interval between doses makes little difference to the result. The blood levels reached did not depend upon the amount of inhibitor given.

In two animals serial determinations of the blood cholinesterase were carried out by a standard manometric method. The total blood cholinesterase, with ACh as substrate, was reduced to less than 5% of normal and did not vary significantly throughout the 5-hr. periods of the experiments.

In another dog the blood ACh was raised to 30 mμg./ml. by giving 5 successive doses of paraoxon. Measured quantities of ACh were then injected intravenously and the blood levels determined immediately, and again 2 hr. later. The blood level after injection corresponded closely to that which would have been expected had the same quantity been added to a volume of blood equivalent to 6% of the body weight. The rate of dis-

TABLE II

BLOOD LEVELS OF ACh (MμG. BASE/ML. WHOLE BLOOD) IN ATROPINIZED DOGS 30 MIN. AFTER SUCCESSIVE DOSES OF PARAOXON

Interval between doses was 45–50 min., except in dog 3, where it was 4 hr. between the 2nd and 3rd doses

Dog No.		Dose Sequence						
		1st	2nd	3rd	4th	5th	6th	Total
1	Paraoxon mg./kg.	3	5	5	10	20	—	43
	ACh mμg./ml.	15	25	20	20	15	—	
2	Paraoxon mg./kg.	3	5	5	5	10	10	38
	ACh mμg./ml.	25	30	30	30	25	20	
3	Paraoxon mg./kg.	3	5	5	10	—	—	23
	ACh mμg./ml.	10	20	20	25	—	—	
4	Paraoxon mg./kg.	3	5	10	10	20	20	68
	ACh mμg./ml.	10	15	20	25	20	20	

appearance from the blood remained slow even when the level had become high (120 mμg./ml.). These findings suggest that the persistent low level of ACh in the blood of dogs poisoned by paraoxon is due to a slower production of ACh rather than to an increased rate of excretion or destruction.

Cats.—Since Douglas and Paton (1951), and Perry (1953), used cats for their observations on ACh output, further experiments were done on these animals. The cats' response to an initial dose of paraoxon (3 mg./kg.) was a failure of respiration similar to that seen in dogs and rabbits ; but a more prolonged period of artificial respiration was usually necessary before natural breathing returned—an average of 17 min., as compared with 2–3 min. in dogs. The response of cats to a second dose of paraoxon was uncertain. Some, like dogs, were unaffected ; others showed further failure of respiration needing prolonged artificial respiration before recovery. Most of the cats died from the effects of the paraoxon, developing circulatory failure in addition to respiratory failure. However, they survived for periods of up to 5½ hr., and observations on the blood ACh levels were made. Cats responded like rabbits (Table III) and showed a progressive rise in the blood ACh. The figures were usually obtained about 30 min. after the paraoxon had been given. On two occasions, when samples were taken 5 min. after the paraoxon, the level was a little higher (by about 5 mμg./ml.) than it was 25 min. later.

The progressive rise of blood ACh only took place when successive doses of paraoxon were given. If these were discontinued the blood level fell slowly, decreasing by about 50% in 2 hr. There is no direct relationship between the total amount of inhibitor given and the final blood level of ACh.

Douglas and Paton (1951) stated that much of the blood ACh in cats poisoned with TEPP came from the intestine. When our experiments were repeated on eviscerated cats a progressive rise of blood ACh took place, but at a reduced rate (Table IV). Two of these cats received smaller initial doses of paraoxon, but otherwise the experiments were comparable to those on normal cats.

In the hope that it might eventually prove possible to analyse blood from individual organs or regions of the body, an attempt was made to assay small samples of blood by the chloralosed cat method. It was found that the doses of atropine normally given to the poisoned cat interfered with the assay.

TABLE III

BLOOD LEVELS OF ACh (MμG. BASE/ML. WHOLE BLOOD) IN ATROPINIZED CATS 30 MIN. AFTER SUCCESSIVE DOSES OF PARAOXON

Interval between doses was 40–45 min., except in cat 3, where it was 2 hr. between 2nd and 3rd doses

Cat No.		Dose Sequence						
		1st	2nd	3rd	4th	5th	6th	Total
1	Paraoxon mg./kg.	3	5	10	13	—	—	31
	ACh mμg./ml.	15	25	45	75	—	—	
2	Paraoxon mg./kg.	3	5	5	5	5	—	23
	ACh mμg./ml.	15	30	35	45	60	—	
3	Paraoxon mg./kg.	3	5	10	10	20	20	68
	ACh mμg./ml.	0	10	20	30	35	45	
4	Paraoxon mg./kg.	3	5	10	10	10	—	38
	ACh mμg./ml.	20	30	40	60	70	—	

TABLE IV

BLOOD LEVELS OF ACh (MμG. BASE/ML. WHOLE BLOOD) IN EVISCERATED ATROPINIZED CATS AFTER SUCCESSIVE DOSES OF PARAOXON

Interval between doses 40–45 min.

Cat No.		Dose Sequence								
		1st	2nd	3rd	4th	5th	6th	7th	8th	Total
1	Paraoxon mg./kg.	1	2	3	5	5	5	5	5	31
	ACh mμg./ml.	—	10	13	20	25	30	45	56	
2	Paraoxon mg./kg.	1	3	5	10	10	10	—	—	39
	ACh mμg./ml.	—	10	15	20	20	40	—	—	
3	Paraoxon mg./kg.	3	5	10	10	10	10	17	—	65
	ACh mμg./ml.	5	7·5	7·5	15	20	25	35	—	

An attempt was made to reduce the amount of atropine to the minimum compatible with survival. The prophylactic atropine was omitted, but a dose of 0.5 mg. was given after the first dose of inhibitor (1 mg./kg.). Further doses of atropine were given as successive doses of paraoxon were injected. To three cats, which received 0.5–1.0 mg./kg. of atropine, a total of 11–16 mg./kg. paraoxon was given.

All the cats died of paraoxon poisoning with blood levels of 20–35 mμg./ml. ACh as determined on the leech. But these levels were in the range at which it was just possible to use the chloralosed cat method of assay ; and, despite the smaller doses of atropine that had been given, enough was still present to invalidate the assay.

Rat Diaphragm.—Perry (1953) found that it was the inhibition of the cholinesterase in the perfused eserinized cat sympathetic ganglion that led to the profound fall in ACh output in response to continued stimulation. The fall did not occur after stimulation in the absence of eserine. The observations on the cat and rabbit poisoned with paraoxon suggested that ACh production might continue after, and be "stimulated" by, repeated doses of the inhibitor. Since it proved impossible to assay the blood from individual organs of the poisoned cat, ACh production in the isolated rat diaphragm was examined. The anaesthetized rat responds like the rabbit or dog to repeated doses of paraoxon, and Stewart (1952) has shown that ACh is present in its blood before death from paraoxon poisoning. Burgen, Dickens and Zatman (1949) showed that the stimulated isolated rat diaphragm will produce ACh in measurable quantities.

When the diaphragm was stimulated at 50/sec. for 20 min. the fluid in the bath contained no ACh. If eserine (5×10^{-5} M) was added, in order to inhibit all the cholinesterase in the preparation, and the diaphragm was stimulated for a period of 20 min., then from 40–100 mμg. of ACh were produced. When four successive periods of stimulation were applied to single preparations there was no consistent or significant decrease in the amount of ACh produced. It made no difference whether the interval between periods of stimulation was 5 min. or 40 min. Some of the figures obtained in these experiments are given in Table V ; it will be seen that eserine and paraoxon gave similar results.

The production of ACh seemed to be steady throughout the period of stimulation, and the amount produced was proportional to the total number of stimuli applied—whether these were in increasing or decreasing order of frequency.

TABLE V

ACh BASE (MμG.) PRODUCED BY A HALF DIAPHRAGM AFTER SUCCESSIVE 20 MIN. PERIODS OF TETANUS AT 50/SEC.

Rest intervals between tetanus periods are either 5 min. or 40 min. Vol. of sample from diaphragm bath = 5 to 7 ml. Max. range of each ACh value = ±25%.

Period of Tetanus	Eserine 5×10^{-5}M						Paraoxon 4×10^{-6}M					
	5′ rest interval			40′ rest interval			5′ rest interval			40′ rest interval		
1st ..	55	50	41	55	54	40	60	30	90	54	52	90
2nd ..	46	55	55	50	50	45	50	32	60	30	62	60
3rd ..	40	57	27	30	50	50	50	0*	50	59	59	43
4th ..	46	46	50	50		50	60	32	50	60	57	65

* Values of 0 were very occasionally recorded with paraoxon, but never with eserine.

Thus the diaphragm produced 22, 35, and 50 mμg. ACh when the sequence of stimulation was 10, 20, and 50 per sec. respectively ; and it produced 65, 32 and 22 mμg. ACh when the sequence was 50, 20, and 10 stimuli per sec.

DISCUSSION

In the anaesthetized rabbit, dog, and cat the failure of a second dose of paraoxon to inhibit respiration and kill the animal is not associated with a lower level of ACh in the blood. In the cat and the rabbit successive doses of paraoxon produce a progressive rise in the blood ACh ; but if no further doses are given the blood level slowly falls, and is reduced by 50% after 2–3 hr. In the dog the ACh level does not continue to rise after the third or succeeding doses of inhibitor ; if no more inhibitor is given the level falls slowly, as in the cat. The dog is much less sensitive to successive doses of inhibitor than is either the cat or the rabbit, though it remains sensitive to injected ACh—which will induce a prolonged and fatal arrest of respiration.

The tolerance of the cat to the circulating ACh is probably related to the amount of atropine administered, which also appears to circulate for some time. The cats given smaller doses of atropine died earlier than those given larger quantities. Protection by atropine is limited, however, and cannot be indefinitely increased by larger doses.

Could the insensitivity of the anaesthetized animal to succeeding doses of paraoxon be due to a falling off in the production of ACh, such as Perry (1953) had shown to take place in the perfused and eserinized sympathetic ganglion of the cat ? In our experiments the level of ACh in the circulation only was measured. This blood ACh is almost certainly an overflow ACh from the tissues, and is the balance between the amount entering the blood stream, and the sum of that

destroyed by any remaining cholinesterase, plus any unchanged ACh which leaves the blood. In two experiments the cholinesterase activity of dogs' blood was consistently less than 5% of normal. The methods of estimation were not accurate enough to determine whether the activity of the cholinesterase of the cat was lower than that of the rabbit. The blood level of ACh does not rise so high in the dog as it does in the rabbit or cat; the dog is also much less sensitive to repeated doses of the inhibitor.

In the absence of further doses of inhibitor the rate of disappearance of ACh from the blood was similar in the three species. Judged by the blood levels, ACh production is most rapid within the first few minutes of giving the inhibitor.

The differences between the cat and rabbit on the one hand, and the dog on the other, could most easily be explained by assuming that the production of ACh is less in the dog; but other explanations are possible.

Death from paraoxon poisoning may be due to excess ACh at certain vital centres whose activities are suppressed or distorted by it. Such an excess may result from a high local production, or from the carriage to such sites of ACh produced elsewhere. Although Douglas and Paton (1951) found the bowel to be an important site for ACh production our observations on eviscerated cats indicate that other tissues produce at least as much as does the gut.

From the work of Perry (1953) it seemed likely that ACh production might fall off very much after prolonged inhibition of cholinesterase such as would follow the large doses of paraoxon given in these experiments. However, in the stimulated isolated diaphragm, ACh production does not fall off, after inhibition of cholinesterase, as it does in sympathetic ganglia. On the hypothesis put forward by Perry to explain his observations one would assume that at sites such as the myoneural junction there was plenty of raw material available for the continuous production of ACh.

On the assumption that death does take place as a result of excess ACh at certain points, an explanation of the findings recorded above, and of those of Perry (1953), can be suggested. At or near certain vital points which are sensitive to ACh, there is an excessive ACh production in response to an initial "lethal" dose of a cholinesterase inhibitor such as paraoxon. If, however, the circulation is maintained by artificial respiration this excess is carried away by the blood, and the animal recovers. But at such sites ACh production falls off rapidly in the presence of continuously inhibited cholinesterase, just as it does in the perfused sympathetic ganglion. Meanwhile ACh production continues at other sites, such as the myoneural junction, and is apparently "stimulated" by each further addition of inhibitor. Production at such sites goes on more rapidly in the rabbit and cat than in the dog, so that in the first two species the blood levels continue to rise until the vital centres are again overwhelmed—this time by ACh brought by the blood. This does not happen in the dog, because its blood ACh never rises as high as that of the cat or rabbit. That the dog is not insensitive to ACh is shown by its response to large doses injected into the blood.

It is difficult to understand the rise of ACh in the blood of cats and rabbits in terms of a true stimulation of production. Nothing is yet known about any such stimulus. It seems probable that a temporary more complete inhibition of cholinesterase takes place. It is not, however, technically possible to measure the small differences in cholinesterase activity that may be important in these conditions.

ACh will protect cholinesterase from inhibition by this type of inhibitor, but to demonstrate this phenomenon it has always been necessary to use concentrations of ACh of an order several times greater than those of the inhibitor (Aldridge, 1950; Augustinsson and Nachmansohn, 1949). This makes it seem unlikely that the protection of any residue of inhibited cholinesterase by ACh is responsible for the insensitivity of the animals to the second and subsequent doses of inhibitor. Were such a mechanism of protection important it would be the cat rather than the dog that would display the greater degree of insensitivity to repeated doses of paraoxon.

Summary

1. The blood level of acetylcholine has been followed in anaesthetized rabbits, dogs, and cats poisoned by the anticholinesterase diethyl-*p*-nitrophenyl phosphate (paraoxon) and kept alive by atropine and short periods of artificial respiration.

2. In the rabbit and cat there is a progressive rise in the blood level of ACh if successive doses of inhibitor are given. If no more inhibitor is given the level falls slowly, to about one half in two hours. Cats and rabbits may die because of respiratory and circulatory failure despite atropine and artificial respiration.

3. In the dog the blood level rises, but not so far as in the cat or rabbit, and the rise is not progressive after repeated doses of inhibitor. The

dog seems to become immune to paraoxon though it remains sensitive to injected ACh. The rate of fall in the absence of further inhibitor is even slower than in the cat or rabbit.

4. The blood ACh in the poisoned eviscerated cat rises progressively though more slowly than in the normal cat.

5. The ACh produced by the stimulated isolated rat diaphragm, in the presence of eserine or paraoxon, remains steady over a number of successive periods of stimulation.

6. These findings are discussed, and an explanation offered for them.

We wish to thank Miss P. Harrison and Mr. C. R. Kennedy for technical assistance, and Mrs. Kemp for carrying out the cholinesterase determinations in dog blood.

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THE INHIBITION OF CHOLINESTERASES BY 3-(DIETHOXYPHOSPHINYLOXY)-*N*-METHYLQUINOLINIUM METHYLSULPHATE AND ITS TERTIARY BASE

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Neutral organic phosphates of the type

$$RO—P(=O)—(OR')_2$$

where R′ represents an alkyl group and R an aryl group are potent inhibitors of cholinesterases. It is now generally assumed that substances of this kind are actually hydrolysed by cholinesterases in the same manner as acetylcholine ; but, because of the great stability of the enzyme-dialkyl phosphate complex which is formed during hydrolysis, their turnover is negligible, and enzyme inhibition is the predominant feature. This " phosphorylation theory " is well supported by two findings ; firstly, within a homologous series of organic phosphates the inhibitory potency is inversely proportional to the stability of the RO–P link (Aldridge and Davison, 1952a and b) ; secondly, the stability of the enzyme-inhibitor complex is a function of the dialkyl phosphate group and is always identical where inhibitors with the same dialkyl phosphate group are used (Burgen and Hobbiger, 1951 ; Wilson, 1952 ; Aldridge, 1953c). Another strong argument in favour of the phosphorylation theory is derived from the results obtained with crystalline chymotrypsin which, like cholinesterase, is also a carboxylic esterase. With chymotrypsin the inhibition by organic phosphates, such as TEPP, DFP, E600 and related compounds, consists of a dialkyl phosphorylation of the enzyme with the liberation of one mole of acid or phenol per mole of enzyme inhibited (Jansen, Fellowes-Nutting, Jang and Balls, 1950 ; Fleischer, Jandorf, Summerson, and Norton, 1950 ; Hartley and Kilby, 1950). Unfortunately, cholinesterase is not yet available in a crystalline form and so accurate quantitative studies of its reaction with anticholinesterases of the organic phosphate type cannot be carried out. All the evidence in favour of the phosphorylation theory is by necessity, therefore, only indirect and studies of the reactions of cholinesterase with new organic phosphates which exceed in potency the known compounds are of considerable value and interest.

In continuation of the work on 3-dialkylphosphato-*N*-dimethylanilines and their quaternary salts (Burgen and Hobbiger, 1951) 3 - (diethoxyphosphinyloxy)-*N*-methylquinolinium methylsulphate became available, and was found to possess an anticholinesterase potency greater than that of any other organic phosphate so far described. The following account describes *in vitro* and *in vivo* experiments with this compound and its tertiary base ; the results agree closely with those previously obtained with other organic phosphates containing the same dialkyl phosphate group (Burgen and Hobbiger, 1951), and thus give additional support to the phosphorylation theory.

METHODS

Cholinesterase activity was determined manometrically at 37° C. in an atmosphere of 95% N_2 and 5% CO_2. The enzyme solutions were placed in the main compartment of the vessel ; substrate and inhibitor, each dissolved in 0.2 ml., were placed in separate side arms. The total volume of fluid was always 3 ml. The final substrate concentrations were: 0.025 M-acetylcholine chloride, 0.03 M-(±)-acetyl-β-methylcholine chloride and 0.01 M-benzoylcholine chloride.

The enzymes used were:

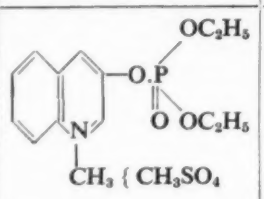
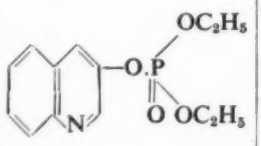
(*a*) From human red cells (twice washed with 0.9% NaCl) and from heparinized human plasma.

(*b*) Purified bovine erythrocyte cholinesterase (Winthrop-Stearns Inc.) and purified human plasma cholinesterase (Plasma fraction IV-6-3 ; prepared in the late Dr. Cohn's laboratory according to the method of Surgenor, Strong, Taylor, Gordon, and Gibson, 1949).

Except when otherwise stated, all experiments were carried out in a buffer solution containing 0.025 M-$NaHCO_3$, 0.075 M-NaCl, 0.075 M-KCl, 0.04 M-$MgCl_2$, and 0.1% crystalline bovine plasma albumin (Armour Laboratories).

Throughout the text the cholinesterase of red cells will be referred to as true cholinesterase and the cholinesterase of plasma as pseudo-cholinesterase.

The anticholinesterases used were:

Code No.	Structural Formula	Chemical Name
Ro 3–0422		3-(diethoxyphosphinyloxy)-*N*-methylquinolinium methylsulphate
Ro 3–0419		3-(diethoxyphosphinyloxy)-quinoline

Both substances are pure compounds, and fresh solutions were made each time before use. Ro 3-0422 is a crystalline, water soluble, hygroscopic salt, whereas Ro 3-0419, the tertiary base, is an oil. Both compounds,* which were synthesized in the Research Department of Roche Products Ltd. by Dr. K. J. M. Andrews, were unstable in solution. At 45° C., 0.1% solutions of Ro 3-0422 and Ro 3-0419, in distilled water, lost 50% of their activity in 67 hours, and in 260 hours respectively.

Tetraethylpyrophosphate (TEPP) was made up as a 1% (w/v) stock solution in dry propylene glycol, and dilutions were made immediately before use.

Results

In vitro *Experiments with 3–0422*

Inhibition as a Function of Inhibitor Concentration, and the Effect of Substrate on Inhibition.—Ro 3–0422 is a potent inhibitor of true and pseudo-cholinesterase. Table I gives the molar concentrations required to produce 50% inactivation of the enzymes under standard conditions. As with most other organic phosphates the slope of the inhibition curve is steep and, on the average, a fourfold difference in the concentration of the inhibitor covers the range from 20 to 80% inhibition. With the substrate concentrations used the inhibition produced by a given concentration of Ro 3–0422 was the same for true cholinesterase when either acetylcholine or acetyl-β-methylcholine was the substrate, and for pseudocholinesterase it was the same with either acetylcholine or benzoylcholine.

With the highly purified enzyme preparations in concentrations roughly equimolar to the unpurified preparations, the amount of the inhibitor required for 50% inhibition was of the same order as that necessary to produce the same degree of inhibition in the crude preparations (Table I).

* The chemistry of these compounds will be published shortly elsewhere.

TABLE I

INHIBITION OF CHOLINESTERASE *IN VITRO* AT 37° C.

The substrate (0·025 M-acetylcholine chloride) was added 20 min. after the inhibitor; the total CO_2 output, between 5 and 35 min. after addition of substrate, was the measure of enzyme activity.

Source of Enzyme and Amount per 3 ml.	Activity in μl. CO_2/10 min. in the Absence of Inhibitor	Molar Concentration of Ro 3–0422 Required for 50% Inhibition
0·06 ml. red cells ..	90·5	$3·3\times10^{-10}$
80 μg. purified true cholinesterase ..	86·6	$3·0\times10^{-10}$
0·2 ml. plasma ..	81·0	$1·2\times10^{-9}$
30 μg. purified pseudocholinesterase ..	76·0	$1·0\times10^{-9}$

If the substrate was added to a solution of true or pseudo-cholinesterase, after the enzyme had been in contact with Ro 3–0422 for 20 minutes, no further inactivation took place so long as the substrate concentration was maintained at a high level. Under the same conditions no enzyme reactivation by substrate occurred (Fig. 1), i.e., the CO_2 output remained constant over at least two consecutive periods of 10 minutes. If, on the other hand, the substrate was added together with, or before, the inhibitor a very marked protection of the enzyme was seen, and this could only be overcome by depletion of substrate or by increasing the concentration of inhibitor (Fig. 1).

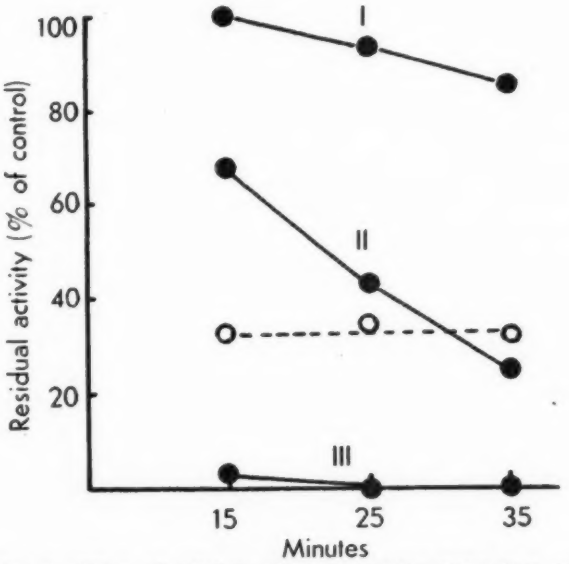


FIG. 1.—Effect of substrate (0.03 M-acetyl-β-methylcholine) on inhibition of true cholinesterase (0.06 ml. washed red cells per 3 ml.). Ordinate: Residual activity as % of control. Abscissa: Time in min. after addition of substrate (each circle gives the activity over the preceding 10 min.). ○ - - - ○: 4.8×10^{-10} Ro 3–0422 added to enzyme 20 min. before substrate. ●——●: Substrate added to enzyme 5 min. before inhibitor. I: 4.8×10^{-10} Ro 3–0422; II: 4.8×10^{-9} Ro 3–0422; III: 4.8×10^{-8} Ro 3–0422. All concentrations of Ro 3–0422 are molar and final.

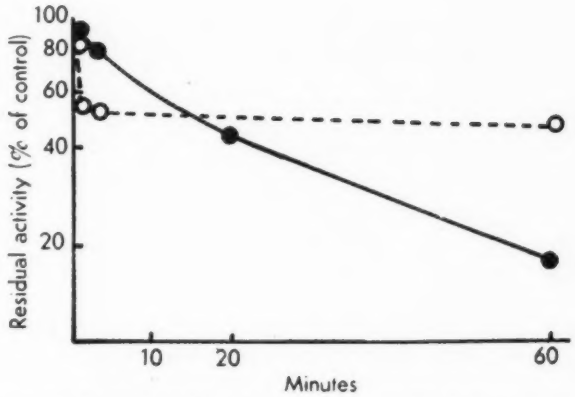


FIG 2.—Rate of combination of Ro 3-0422 with cholinesterase at 37° C. ●——●: 60 μg./3 ml. purified true cholinesterase in a 3×10^{-10} M solution of Ro 3-0422. ○ - - - ○: 30 μg./3 ml. purified pseudo-cholinesterase in a 1×10^{-9} M solution of Ro 3-0422. Ordinate: Residual activity as % of control. Abscissa: Time allowed for contact of Ro 3-0422 with enzyme before addition of substrate.

From these experiments it can be concluded that Ro 3-0422 competes with ACh for the same part of the enzyme surface; but, because of the relatively stable nature of the enzyme-inhibitor complex, the competitive nature of this reaction cannot be demonstrated when the substrate is added to the enzyme after the inhibitor.

Rate of Combination with Cholinesterases.—The inhibition of cholinesterases by organic phosphates (except those containing a dimethyl group) shows the characteristics of a bimolecular reaction with one component—the inhibitor—in excess (Aldridge and Davison, 1952a and b). With Ro 3-0422 the results were as follows:

When purified true cholinesterase was incubated with Ro 3-0422 a progressive inactivation of the enzyme took place; but, as can be seen from Fig. 2, the rate of enzyme inactivation showed a decrement with time which was greater than that which would be expected from a first order reaction. With purified pseudo-cholinesterase this was even more marked and, with a given concentration of Ro 3-0422, inhibition reached a maximum value within the first two minutes (Fig. 2). The same results were obtained with crude enzyme preparations.

One possible reason for the decrement in the rate of inhibition is that the inhibitor may undergo spontaneous hydrolysis. This, however, can be excluded because of the difference in behaviour of true and pseudo-cholinesterase and also by the fact that a 5×10^{-9} molar solution of Ro 3-0422 in buffer loses 50% of its activity at 37° C. only after 18 hours.

Another reason for the inexplicable time course of inhibition—especially of pseudo-cholinesterase—could be the enzymatic destruction of the inhibitor by a phosphatase. This point was followed up and was also excluded.

Enzymatic Hydrolysis of Ro 3-0422.—When the enzymatic hydrolysis of a 3×10^{-3} M solution of Ro 3-0422 was determined manometrically in the usual buffer solution no hydrolysis of the ester was detectable with crude or purified true cholinesterase, or purified pseudo-cholinesterase. Human plasma, on the other hand, metabolized the ester at a slow rate and 50% of the total hydrolysis was achieved in about 200 min. When $MgCl_2$ was omitted from the buffer solution, or when the experiments were carried out in a medium consisting only of 0.025 M-$NaHCO_3$, the activity of human plasma was increased about tenfold; no activity, however, could be detected under the same conditions with purified true or pseudo-cholinesterase or with crude true cholinesterase.

With any given sample of human plasma the rate of hydrolysis was characteristic of a first order reaction (Fig. 3). Using plasma taken from different persons or animals the rate of hydrolysis of Ro 3-0422 varied considerably and no correlation between cholinesterase activity and phosphatase activity could be found.

This enzymatic hydrolysis of the ester is affected most strongly by *p*-chloromercuribenzoic acid. Table II lists all the inhibitors studied.

These results closely resemble Aldridge's for the enzymatic hydrolysis of E600 and its dimethyl homologue (Aldridge, 1953a, b, and c), and it may

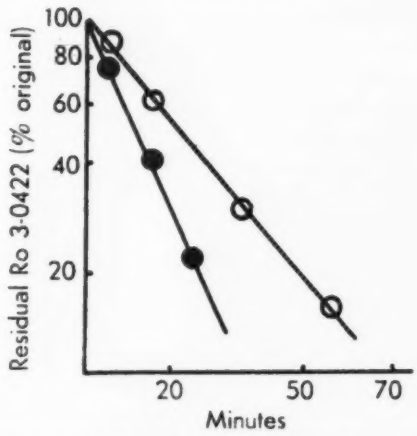


FIG. 3.—Hydrolysis of 3×10^{-3} M sol. of Ro 3-0422 by 0.2 ml. heparinized human plasma/3 ml. Ordinate: Residual Ro 3-0422 as % of original amount. Abscissa: Time in min. ●——●: Subject I; cholinesterase activity of the same sample 292 μl. CO_2/10 min. ○——○: Subject II. Cholinesterase activity of the same sample = 202 μl. CO_2/10 min.

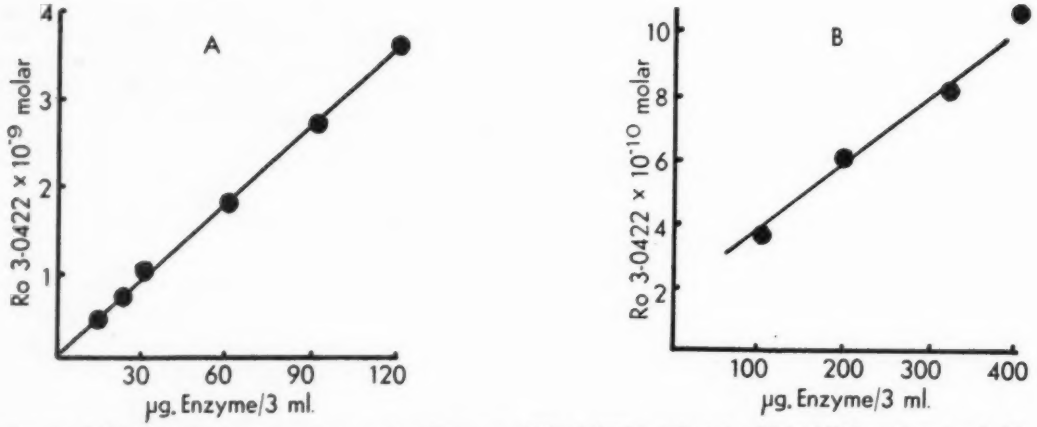


FIG. 4.—Relation between enzyme conc. and molar conc. of Ro 3–0422 required for 50% inhibition of enzyme in 20 min. at 37° C. A: purified pseudo-cholinesterase. B: purified true cholinesterase. Ordinate: molar concentration of Ro 3–0422. Abscissa: µg. of enzyme/3 ml.

TABLE II

INHIBITION OF THE ENZYMATIC HYDROLYSIS OF A 3×10^{-5}M SOLUTION OF Ro 3–0422

By 0·2 ml. of heparinized human plasma in a total volume of 3 ml. (medium: 0·025 M-$NaHCO_3$). All determinations were manometric.

	Molar Concentration	Time Required for 50% Hydrolysis in Min.	% Inhibition
Control	—	19	—
p-Chloromercuri-benzoic acid ..	10^{-4}	335	95
	10^{-5}	20	5
Nickel sulphate ..	10^{-4}	37	49
	10^{-5}	19	0
Copper sulphate ..	10^{-4}	32	41
	10^{-5}	20	5
Potassium oxalate ..	10^{-2}	136	86
	10^{-3}	19	0
Beryllium sulphate ..	10^{-2}	63	70
Sodium cyanide ..	10^{-2}	20	5
„ fluoride ..	10^{-2}	5	Activation

be that the same type of enzyme (called A-esterase by Aldridge) is responsible.

Effect of Enzyme Concentration on Inhibition.—It was shown by Nachmansohn, Rothenberg, and Field (1948) and Bain (1949) that the inhibition of cholinesterase by TEPP and DFP is not only a function of the inhibitor concentration but also of the enzyme concentration. The same applies to the inhibition of cholinesterases by Ro 3–0422. Fig. 4 shows that with both cholinesterases the amount of the inhibitor required for a 50% inhibition of the enzyme is a linear function of the enzyme concentration.

The ratio

$$\frac{[I]_{50} \text{ for enzyme concentration } 2x}{[I]_{50} \text{ for enzyme concentration } x}$$

is 1.63 for true cholinesterase and 2.0 for pseudo-cholinesterase. Assuming that only 1 inhibitor molecule combines with 1 enzyme centre, we can conclude from these figures that the molar concentration of active enzyme centres, in a preparation which, under the experimental conditions used in these experiments, has an activity of 100 µl. CO_2/3 ml./10 min. (substrate: 0.025 M-acetylcholine chloride), cannot be greater than 2.4×10^{-9} for pseudo-cholinesterase, and must be less than 6×10^{-10} for true cholinesterase.

Inhibition of Cholinesterase in Mixtures Containing True and Pseudo-Cholinesterases.—If the above figures approach the true values (which cannot be worked out at present because of the lack of a sufficiently pure enzyme preparation) the specificity of Ro 3–0422 as shown in Table I might not be a true one but might be a reflection of differences in the enzyme concentration. This point was therefore reinvestigated using mixtures of true and pseudo-cholinesterase instead of a single enzyme. Under such conditions, as Table III shows, the results are the reverse of those presented in Table I; Ro 3–0422 has thus to be con-

TABLE III

INHIBITION OF PURIFIED TRUE AND PSEUDO-CHOLINESTERASE, SINGLY AND IN A MIXTURE

The substrate (0·03 M-acetyl-β-methylcholine and 0·01 M-benzoylcholine) was added 20 min. after the inhibitor in a final concentration of 8×10^{-10} M; the total CO_2 output between 5 and 35 min. after addition of substrate was the measure of enzyme activity.

Enzyme and Amount per 3 ml.	Activity (as % of Control) Determined by Hydrolysis of	
	Acetyl-β-methylcholine	Benzoylcholine
80 µg. purified true cholinesterase	12	—
30 „ „ pseudo-cholinesterase	—	60
80 „ „ true cholinesterase plus 30 „ „ pseudo-cholinesterase	93	65

sidered an inhibitor with a higher affinity for pseudo- than for true cholinesterase.

Stability of the Enzyme-inhibitor Complex.—The stability of an enzyme-inhibitor complex which is formed between cholinesterase and organic phosphates with a diethyl phosphate or di*iso*propyl phosphate group is of an order which makes it impossible to detect enzyme recovery in short-lasting experiments or in the presence of free inhibitor. Enzyme recovery after inhibition, however, can be demonstrated either by allowing the enzyme-inhibitor complex to react with nucleophilic reagents (Wilson, 1951 and 1952) or by measuring enzyme activity at daily intervals after removal of the free inhibitor (Hobbiger, 1951; Burgen and Hobbiger, 1951; and Davison, 1953). The latter procedure was used to study the stability

TABLE IV

TIME COURSE OF REACTIVATION OF TRUE CHOLINESTERASE *IN VITRO* AFTER REMOVAL OF THE UNCOMBINED INHIBITOR AT 37° C.

Intact human red cells were suspended in a 0·9% NaCl soln. containing the inhibitor and kept at room temperature for 30 min. The excess of uncombined inhibitor was then removed by centrifugation and repeated washing of the red cells with fresh saline; 1 ml. of packed red cells was then haemolysed in 49 ml. 0·025 M-$NaHCO_3$ and the haemolysate incubated at 37° C. The cholinesterase activity was measured shortly after haemolysis, and again after 24 and 48 hr. incubation, by taking a small sample each time from the incubated solution. The substrate was 0·03 M-acetyl-β-methylcholine.

Inhibitor	After Removal of Uncombined Inhibitor		After 24 hr.		After 48 hr.	
	μl. CO_2/10 min.	Activity (% of Control)	μl. CO_2/10 min.	Activity (% of Control)	μl. CO_2/10 min.	Activity (% of Control)
Exp. I:						
Control	39·5	100	36·9	93	37·5	95
TEPP (10^{-6}M)	3·3	8	11·9	32	18·0	48
Ro 3–0422 (10^{-7}M)	2·9	7	9·4	26	17·7	47
Exp. II:						
Control	47·2	100	45·5	88	47·2	81
TEPP (10^{-6}M)	0·0	0	8·5	18	17·5	37
Ro 3–0422 (10^{-5}M)	1·9	4	9·0	19	16·1	34

of the enzyme-inhibitor complex formed between true cholinesterase and Ro 3–0422. As Table IV shows, Ro 3–0422 cannot be classified as an irreversible inhibitor like DFP, but forms an enzyme-inhibitor complex which has the same stability as that of cholinesterase and TEPP. As the results were the same with crude and purified enzyme preparations we can exclude the possibility that the enzyme recovery shown in Table IV is significantly affected by an action of a second enzyme—such as, for example, a phosphatase.

These results agree well with those previously described for other organic phosphates containing the same dialkyl phosphate group; they must, therefore, be regarded as affording additional support for the phosphorylation theory.

The *in vitro* results on the stability of the enzyme-inhibitor complex are paralleled by the *in vivo* recovery of cholinesterase activity of red cells in rabbits given a single injection of Ro 3–0422. With a dose that reduced enzyme activity by 90% the activity returned to normal within four days, reaching a level of 44% after 24 hours, 62% after 48 hours and 90% after 72 hours. This is in agreement with what happens after an injection of TEPP, or other organic phosphates containing the same dialkyl phosphate group.

In vivo *Experiments with Ro 3–0422*

The parenteral administration of Ro 3–0422 produced effects in rats, rabbits, and cats which could be fully attributed to its action upon cholinesterase. The first observable changes were muscarinic—salivation, lacrimation, and bradycardia. When the dose was increased nicotinic effects—fasciculations and twitchings—were seen in addition.

The intravenous LD50 in mice was 20 μg./kg. Ro 3–0422 is, therefore, more toxic than any other organic phosphate; this was to be expected from its high anticholinesterase activity *in vivo* (Jones, Meyer and Karel, 1948).

In cats, intravenous doses of 0.1–1 μg./kg. were sufficient to potentiate and prolong the actions of injected ACh and of vagal stimulation, and to produce bradycardia. With 5 μg./kg. salivation was abundant, micturition occurred, intestinal activity was increased, fibrillary muscular twitchings were seen, and the response of skeletal muscles to indirect stimulation was markedly potentiated.

On the isolated rat diaphragm a dose as small as 0.01 μg./ml. potentiated twitch tension, and on the isolated rabbit intestine an increase in tone and spontaneous peristalsis occurred at similar dose levels.

In studies on the distribution of Ro 3–0422 *in vivo* (judged by the inhibition produced at various sites after parenteral administration) the results closely resembled those with Ro 3–0340 (Burgen and Hobbiger, 1951). Thus at low doses of the inhibitor the inhibition of blood cholinesterases was more marked than was that of tissue cholinesterases; but if the dose was increased a state of uniform inhibition was achieved. Again, as with the quaternary salts of 3-dialkylphosphato *N*-dimethylanilines, the brain was never inhibited even after a lethal dose of the inhibitor.

Action of Ro 3–0419, the Tertiary Base of Ro 3–0422

The tertiary base of Ro 3–0422 is as potent as the latter in inhibiting pseudo-cholinesterase but less active against true cholinesterase. Under standard conditions, such as those which are given in Table I, the molar concentration of Ro 3–0419 required for 50% inhibition was 3.8×10^{-9} for pseudo-cholinesterase and 7.8×10^{-8} for true cholinesterase. With both enzymes the reaction between enzyme and inhibitor followed the time course of a first order reaction, and no enzymatic destruction of the inhibitor by plasma or any other of the enzyme preparations was noticed. The inhibition of cholinesterase was competitive with substrate, and the substrate gave marked protection. Enzyme recovery *in vitro* and *in vivo* was similar to that after inhibition by TEPP and by Ro 3–0422.

The intravenous LD50 in mice was 1 mg./kg. In contrast to Ro 3–0422, brain cholinesterase was not spared from inhibition when the inhibitor was injected into an animal; the degree of inhibition of brain cholinesterase after a given dose of the inhibitor was of the same order as that of blood cholinesterases.

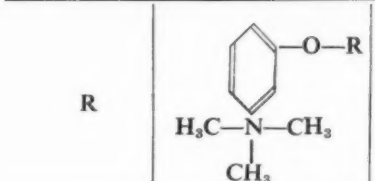
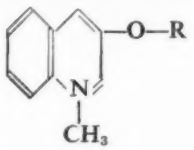
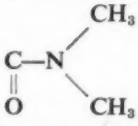
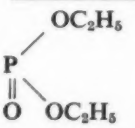
Discussion

The potency of an anticholinesterase of the organic phosphate type depends on certain structural features of the inhibitor molecule, and on the reactivity of the RO–P bond (Mackworth and Webb, 1948; Brauer, 1948; Aldridge and Davison, 1952a and b; Aldridge, 1953c). As would be expected, both these points play a significant part in determining the anticholinesterase potency of Ro 3–0422. Table V shows the concentration required for 50% inhibition of true cholinesterases for the 3-hydroxy, 3-dimethylcarbamoyloxy and 3-diethylphosphato derivatives of trimethylanilinium and methylquinolinium salts. As can be seen from this Table, the introduction of a dimethyl carbamate group leads, in both series, to a comparable gain in activity. This would be expected, since with the dimethylcarbamates two points of attachment are available (quaternary ammonium group and carbamate group) against the one point of attachment (quaternary ammonium group) in the parent substances. If the dimethyl carbamate group in both series is replaced by a diethyl phosphate group, only the phosphate in the methylquinolinium series shows a gain in activity over its corresponding dimethylcarbamate. This cannot be explained on the basis of the structural features of the two phosphates,

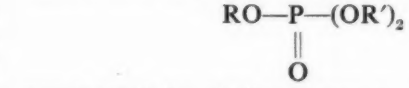
Table V

COMPARISON OF THE ANTICHOLINESTERASE POTENCY OF A SERIES OF TRIMETHYLANILINIUM AND METHYLQUINOLINIUM DERIVATIVES

The unbracketed numerals in this table are the molar concentrations of inhibitor required for 50% inhibition. Enzyme from 0·06 ml. washed human red cells. Inhibitor added to enzyme 20 min. before substrate (0·025 M-acetylcholine); the CO_2 output between 5 and 35 min. after addition of the substrate was the measure of enzyme activity

R		
H	$1{\cdot}7 \times 10^{-4}$ (Ro 2–2561)	$2{\cdot}7 \times 10^{-3}$ (Ro 3–0472)
	6×10^{-8} (Neostigmine)	6×10^{-6} (Ro 1–5384)
	8×10^{-8} (Ro 3–0340)	$3{\cdot}3 \times 10^{-10}$ (Ro 3–0422)

since the changes in both series are identical. The only difference is found in the RO–P bond, which in Ro 3–0422 is much less stable than in Ro 3–0340. If Ro 3–0422 only formed a simple addition complex with cholinesterase this liability of the ester link would reflect itself in a considerable decrease —and not an increase—in activity. The results shown in Table V are strongly in favour of the theory that organic phosphates of the type



are treated by cholinesterase in the same manner as substrates but differ from the latter only in the stability of the enzyme-dialkyl phosphate complex.

It has been pointed out in a previous paper (Burgen and Hobbiger, 1951) that if the phosphorylation theory is correct the stability of the enzyme-inhibitor complex should be the same whenever the inhibitor has the same dialkyl phosphate group. With cholinesterase inhibited by Ro 3–0422, or its tertiary homologue Ro 3–0419, the stability of the enzyme-inhibitor complex is of the same order as that formed between cholinesterase and TEPP, or Ro 3–0340. All these four inhibitors have in common a diethyl phosphate group, and so the results presented in this paper fully support the phosphorylation theory.

Although Ro 3–0422 is an inhibitor of great potency it is not at present possible to say what is the quantitative relationship of the reaction between enzyme and inhibitor. The data available from the literature on the turnover of substrate by cholinesterase provide only approximate figures, since they have either been obtained indirectly from work with inhibitors (Easson and Stedman, 1936; Goldstein, 1944; Myers, 1951; and Berry, 1951) or apply to work done with cholinesterase from a non-mammalian source (Rothenberg and Nachmansohn, 1947). Until compounds of the type described by Funke, Depierre and Krucker (1952), which inhibit cholinesterase in 10^{-14} and 10^{-16} molar concentrations, have been studied more extensively, and until a pure enzyme preparation of cholinesterase is available, it will not be possible to interpret the reaction between enzyme and Ro 3–0422 on a quantitative basis.

Its high anticholinesterase activity makes Ro 3–0422 very suitable for an *in vivo* analysis of the function of the acetylcholine-cholinesterase system. If the compound is used for such a purpose it must be remembered that the distribution on a cellular level will be affected by the physicochemical properties of the substance (Ro 3–0422 is not lipoid soluble) and could also reflect enzymatic destruction of the inhibitor by an enzyme which has all the characteristics of the A-esterase described by Aldridge (1953a and b).

Summary

1. 3-(Diethoxyphosphinyloxy)-*N*-methylquinolinium methylsulphate (Ro 3–0422) is the most potent anticholinesterase so far synthesized. It inhibits true cholinesterase in concentrations as low as 10^{-10} molar. With true and pseudocholinesterase the degree of enzyme inhibition is a function of enzyme and inhibitor concentration.

2. The enzyme-inhibitor complex formed between cholinesterase and Ro 3–0422 *in vivo* and *in vitro* is only slowly reversible and shows the same stability as the enzyme-inhibitor complex formed between cholinesterase and other diethyl phosphates such as TEPP.

3. Crude human plasma contains an enzyme (probably the A-esterase described by Aldridge) which hydrolyses Ro 3–0422 in the concentrations required to inhibit cholinesterase.

4. Ro 3–0422 is more toxic than any other organic phosphate and its LD50 in mice is 20 μg./kg. Doses below 1 μg./kg. produce in cats the typical symptoms of cholinesterase inhibition.

5. Ro 3–0419, the tertiary base of Ro 3–0422, is a weaker anticholinesterase than is Ro 3–0422.

6. The results obtained with Ro 3–0422 and Ro 3–0419 fully support the theory that inhibition of cholinesterase by organic phosphates is a dialkyl phosphorylation of the enzyme.

I wish to thank Professor C. A. Keele, Professor F. Bergel, and Dr. A. L. Morrison for their helpful discussions, and in addition I am grateful to Professor F. Bergel and Dr. A. L. Morrison for a generous supply of all the organic phosphates used in this work. To the late Dr. E. J. Cohn I am indebted for a gift of purified pseudo-cholinesterase (human plasma fraction IV-6-3).

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FURTHER STUDIES ON COMPOUNDS REDUCING SYNOVIAL MEMBRANE PERMEABILITY

BY

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(RECEIVED NOVEMBER 24, 1953)

It has been shown (Bianchi, 1953) that benzoylcarbinol acetate decreases synovial permeability in the talo-crural articulation of the rabbit, but not the permeability of periarticular subcutaneous tissue, capillaries, or renal tubules. The results obtained with other compounds related to benzoylcarbinol acetate are reported in this paper with a view to increasing our knowledge of compounds which could be used in human arthritic processes.

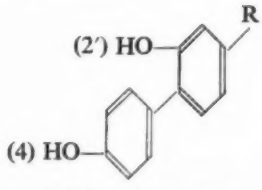
The method used is that first described by Seifter, Baeder, and Begany (1949). These authors consider it useful for the evaluation of new anti-arthritic compounds (Seifter, Baeder, Begany, Rosenkranz, Djerassi, Pataki, and Kaufmann, 1950), but this opinion is not accepted by others (Paul, Hodges, Knouse, and Wright, 1952; Hidalgo, McClure, Henderson, Whitehead, and Smyth, 1952). Human synovial membrane permeability is also known to be modified by drugs (Fitch, Warter, Seifter, Jallo, and Corn, 1950). Seifter's test only gives information on synovial membrane permeability, and not on the total and more complex anti-rheumatic effect of a drug. Only a study of the activity of new drugs in human arthritis, as compared with their effects on the permeability of synovial membrane of laboratory animals, could establish the value of Seifter's test.

METHODS

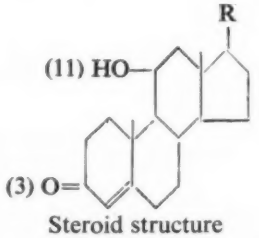
Male rabbits weighing about 2 kg. were treated for four days with a 2% (w/v) concentration of the drugs suspended in a 10% (w/v) gum acacia solution. The test products were usually injected intraperitoneally (75 mg./kg. per day) for four days. About 1 hr. after the last injection, the rabbits were anaesthetized intravenously with 40 mg./kg. 5-5′*iso*amylethylbarbituric acid ("Ethamyl," Zambeletti). A soft rubber catheter was inserted through the urethra into the bladder. In the first group of experiments 0.25 ml. of a 0.5% (w/v) phenol red solution was injected into the right talo-crural articulation. In the second group of experiments the same quantity of phenol red was injected into the periarticular connective tissue. In the third group of experiments 5 ml. of a 0.025% (w/v) phenol red solution was injected intravenously. The recovery of phenol red in the urine was assessed 1, 2, and 3 hr. after the intra-articular, periarticular, or intravenous injection. Details of the methods used are given in a previous paper (Bianchi, 1953).

The chemical properties of the compounds tested are described elsewhere (Logemann and Giraldi, 1951; Logemann, 1952; Logemann and Giraldi, 1953). The compounds are divisible into four groups: (1) Those with a keto-alcoholic side chain. (2) Those derived from dioxyacetone. (3) Those derived from 2′: 4-dioxydiphenyl. (4) Miscellaneous compounds.

The compounds of group 1 have the same side chain as DOCA. Those of group 2 have the same side chain as cortisone. Those of group 3 have two hydroxy groups in positions analogous to the 3: 11 positions of steroid compounds.



Diphenyl structure



Steroid structure

In group 4 are derivatives of acetophenone, one cyclic keto-alcohol (inosose), the therapeutic agent phenylbutazone ("butazolidin"), and sodium benzoate. The last-named compound was tested because Bernini (1953) proved that nearly 50% of benzoylcarbinol is excreted as hippuric acid. It is known that sodium benzoate is excreted as hippuric acid. It was interesting to ascertain whether benzoic acid, presumably produced in the metabolism of benzoylcarbinol, had any action on synovial permeability.

RESULTS

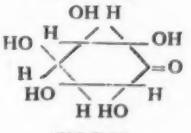
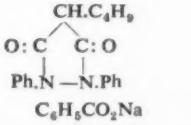
Renal excretion of phenol red 1 and 3 hr. after intra-articular injection is shown in Table I.

The most active of the compounds tested may be arranged, in descending order of their activity in reducing the permeability of the synovial

TABLE I

RENAL EXCRETION OF PHENOL RED INJECTED INTRA-ARTICULARLY. MALE RABBITS TREATED INTRAPERITONEALLY FOR 4 DAYS (75 MG./KG. PER DAY) WITH THE COMPOUNDS INDICATED

(For amount of phenol red injected, see text. The amount excreted is the % of that injected ± S.E.)

Compd. No.	Chemical Name	Structural Formula	Phenol Red Excreted After		No. of Animals Used
			1 hr.	3 hr.	
	Compounds of General Formula: R—CO—CH_2OH				
	Controls		53·4±1·4	76·7±1·5	22
1	Benzoylcarbinol	$C_6H_5CO.CH_2OH$	32·3±2·3	61·6±2·3	8
2	*N*-β-Naphthylglycylcarbinol	β-$C_{10}H_7NH.CO.CH_2OH$	37·5±4·6	70·6±4·7	6
3	*N*-α-Naphthylglycylcarbinol	α-$C_{10}H_7NH.CO.CH_2OH$	45·7±5·4	69·2±6·2	4
4	Benzoylcarbinol acetate	$C_6H_5CO.CH_2O.CO.CH_3$	38·2±3·6	62·2±2·2	7
5	Benzoylcarbinol salicylate	$C_6H_5CO.CH_2O.CO.C_6H_4OH$	35·8±2·7	59·8±5·6	5
6	Benzoylcarbinol propionate	$C_6H_5CO.CH_2O.CO.C_2H_5$	46·4±9·4	69·3±10·4	4
7	4-Acetoxybenzoylcarbinol acetate	4-$AcC_6H_4CH_2O.CO.CH_3$	21·9±4·4	50·5±3·4	6
8	4-Styrylbenzoylcarbinol acetate	4-$C_6H_5CH:CH.C_6H_4CO.CH_2O.CO.CH_3$	37·5±5·1	59·2±6·7	7
9	4-Phenylbenzoylcarbinol acetate	4-$C_6H_5C_6H_4CO.CH_2O.CO.CH_3$	34·0±3·9	65·6±2·6	4
10	1(4′-Diphenylyl)-3-acetoxyacetone	4-$C_6H_5C_6H_4CH_2CO.CH_2O.CO.CH_3$	49·3±3·5	79·1±1·2	4
11	4-Methoxybenzoylcarbinol acetate	4-$MeO.C_6H_4CO.CH_2O.CO.CH_3$	43·2	79·1	2
12	β-6-Methoxynaphthoylcarbinol acetate	β-6-$MeO.C_{10}H_6CO.CH_2O.CO.CH_3$	56·5±3·5	76·9±5·9	3
13	β-6-Acetoxynaphthoylcarbinol acetate	β-6-$AcO.C_{10}H_6CO.CH_2O.CO.CH_3$	41·7±3·0	68·9±3·6	7
	Compounds of General Formula: R—C(OR″)(R′)—CO—CH_2—OH				
14	*O*-Acetylmandeloylcarbinol	$C_6H_5CH(OAc)CO.CH_2OH$	30·0±5·2	62·9±4·5	5
15	1(4′-Diphenylyl)-1-methyldioxyacetone-1-methylcarbonate	4′-$C_6H_5C_6H_4C(O.CO_2Me)(Me)CO.CH_2OH$	45·2±6·8	73·4±5·8	4
16	1(4′-Diphenylyl)dioxyacetone-1-methylcarbonate	4′-$C_6H_5C_6H_4CH(O.CO_2Me)CO.CH_2OH$	40·7±5·5	68·2±4·3	4
	Compounds of General Formula: R—C6H4—C6H3(R)—$CO.CH_2O.COCH_3$				
17	4: 2′-Dimethoxy-4′-acetoxyacetonyldiphenyl	4-$MeOC_6H_4C_6H_3$(OMe(2′))(CH_2COCH_2OAc(4′))	47·7±6·0	75·7±3·7	4
18	4(4′-Methoxyphenyl)-3-methoxybenzoylcarbinol acetate	4(4′-$MeOC_6H_4$)C_6H_3(OMe(3))($CO.CH_2OAc$(1))	51·6±2·9	78·7±2·6	4
19	4(4′-Acetoxyphenyl)-3-acetoxybenzoylcarbinol acetate	4(4′-$AcOC_6H_4$)C_6H_3(OAc(3))($CO.CH_2OAc$(1))	53·0±6·3	78·4±2·7	4
	Miscellaneous Compounds				
20	Acetophenone	$C_6H_5CO.CH_3$	43·5±3·1	63·5±1·5	5
21	ω-Amino-acetophenone	$C_6H_5CO.CH_2NH_2$	46·5±6·1	74·1±2·0	4
22	*N*-Acetyl-4-nitro-ω-amino-acetophenone	4-$NO_2C_6H_4CO.CH_2NH.CO.CH_3$	47·7±5·2	74·9±2·9	4
23	2-Acetamino-3-hydroxy-propiophenone	$C_6H_5CO.CH(CH_2OH)(NH.CO.CH_3)$	44·5±1·9	77·7±1·5	4
24	Inosose		52·8±7·8	85·7±5·2	5
25	4-Butyl-1-2-diphenyl-3-5-diketopyrazolidine (Phenylbutazone, "butazolidin")		39·3±2·5	66·3±2·6	6
26	Sodium benzoate	$C_6H_5CO_2Na$	42·8±5·0	68·7±3·7	7

membrane (as measured by the elimination of phenol red 1 hr. after its injection), as follows: 4-acetoxybenzoylcarbinol acetate (No. 7); *O*-acetylmandeloylcarbinol (No. 14); benzoylcarbinol (No. 1); 4-phenylbenzoylcarbinol acetate (No. 9); benzoylcarbinol salicylate (No. 5); 4-styrylbenzoylcarbinol acetate (No. 8); *N*-β-naphthylglycylcarbinol (No. 2); benzoylcarbinol acetate (No. 4); phenylbutazone (No. 25); 1-diphenylyldioxyacetone-1-methylcarbonate (No. 16).

It seems that greater inhibiting action is associated with the structural group R—CO—CH_2OH. Substitution of the alcoholic group with H or NH_2 decreases activity. The compounds most active in decreasing the renal excretion of phenol red injected intra-articularly were also tested for their action on subcutaneous tissue. The results obtained are shown in Table II. Renal excretion of phenol red injected subcutaneously was decreased by benzoylcarbinol salicylate and by phenylbutazone.

TABLE II

RENAL EXCRETION OF PHENOL RED INJECTED SUBCUTANEOUSLY. MALE RABBITS TREATED INTRAPERITONEALLY FOR 4 DAYS (75 MG./KG. PER DAY) WITH THE COMPOUNDS INDICATED

(For amount of phenol red injected, see text. The amount excreted is the % of that injected ± S.E.)

Compd. No.	Chemical Name	Phenol Red Excreted After		No. of Animals Used
		1 hr.	3 hr.	
	Controls	55·4±3·5	80·7±3·1	15
1	Benzoylcarbinol ..	52·9±2·8	86·8±1·7	5
4	Benzoylcarbinol acetate	58·3±4·6	84·8±3·8	4
5	Benzoylcarbinol salicylate	37·7±4·4	78·7±1·7	8
7	4-Acetoxybenzoylcarbinol acetate ..	53·1±5·1	84·8±3·1	4
25	Phenylbutazone ..	36·6±5·7	69·2±5·4	5

TABLE III

RENAL EXCRETION OF PHENOL RED INJECTED INTRAVENOUSLY. MALE RABBITS TREATED INTRAPERITONEALLY FOR 4 DAYS (75 MG./KG. PER DAY) WITH THE COMPOUNDS INDICATED

(For amount of phenol red injected, see text. The amount excreted is the % of that injected ± S.E.)

Compd. No.	Chemical Name	Phenol Red Excreted After		No. of Animals Used
		1 hr.	3 hr.	
	Controls	73·6±2·0	89·0±2·0	20
5	Benzoylcarbinol salicylate	58·7±3·4	80·3±2·4	6
25	Phenylbutazone ..	63·4±5·0	82·1±3·8	6

This test does not exclude the possibility of some renal impairment as a factor in the results.

The results on renal excretion are shown in Table III. Benzoylcarbinol salicylate alone slightly decreases renal excretion of intravenously injected phenol red; phenylbutazone does not.

DISCUSSION

The results cited in this paper call attention to studies on synovial permeability, because it is evident that there are some simple chemical compounds which have a specific "antipermeability" action on synovial membranes. Clinical trials on compounds which are active on the rabbit synovial membrane might indicate the value of this method as an anti-arthritic screening test.

The results already published give some indication as to the mode of action of these compounds. Bianchi and Meli (1952) showed that, when injected into the synovial cavity, 4-acetoxybenzoylcarbinol acetate is devoid of activity on synovial membrane permeability. Capraro and Meli (1951) and Bianchi and Meli (1952) reported on the anti-hyaluronidase action of these compounds. Cresseri and Meli (1953) observed that benzoylcarbinol and benzoylcarbinol acetate do not increase the survival of bilaterally adrenalectomized rats; do not protect mice against the toxic effects of KCl; do not affect liver glycogen deposition in bilaterally adrenalectomized rats, or the adrenals and body weight of normal rats. Benzoylcarbinol on the contrary has, like cortisone, a strong inhibitory action on the granulomatous tissue. Linnell and Roushdi (1941) observed that benzoylcarbinol acetate has very little activity compared to that of DOCA on the survival of bilaterally adrenalectomized rats (1/2,500). De Caro and Cappelli (1953) observed that the adrenal weight of unilaterally adrenalectomized rats increased significantly after treatment with benzoylcarbinol, benzoylcarbinol acetate, and 4-phenylbenzoylcarbinol acetate.

Many of the reported data are in agreement with a direct action of these compounds. But the absence of an inhibitory action of 4-acetoxybenzoylcarbinol acetate injected intra-articularly, and the data of De Caro *et al.* on unilaterally adrenalectomized rats, give the impression that these substances also act by some intermediate mechanism, presumably through stimulation of the hypophysis-adrenal system.

SUMMARY

1. Many compounds related to benzoylcarbinol decrease synovial permeability in the talo-crural articulation of the rabbit but not the permeability of periarticular subcutaneous tissue or excretion by the renal tubules.

2. 4-Acetoxybenzoylcarbinol acetate has a greater action than benzoylcarbinol.

3. Phenylbutazone decreases the permeability of the synovial membrane and the periarticular tissue, but does not impair renal function.

I am indebted to Mr. Gianfranco Biganzoli for his technical assistance.

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Brit. J. Pharmacol. (1954), **9**, 170.

DEPRESSION OF AUTONOMIC GANGLIA BY BARBITURATES

BY

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It is widely known that the administration of the barbiturates may be followed by lowering of the arterial blood pressure, both in laboratory animals (Impens, 1912; Gruber and Roberts, 1926) and in man (Mason, Baker, and Pilcher, 1930; Emge and Hoffman, 1930).

This fall in blood pressure induced by barbiturates is generally ascribed to a depression of the hypothalamic centres (Leiter and Grinker, 1934; Masserman, 1937), and the possibility of simultaneous depression of the peripheral autonomic system seems to have received little consideration.

The present work arose from consideration of the possibility that depression of vasomotor ganglia might contribute substantially to the vasodepressor effect of these drugs. On the excised sympathetic ganglion, depressant effects of pentobarbitone have been investigated by Heinbecker and Bartley (1940), Larrabee, Ramos, and Bülbring (1950), and by Larrabee and Posternak (1952). Paralysis of the superior cervical ganglion of the cat, during perfusion with fluids containing a barbiturate, is described by Brücke, Macho, and Werner (1947).

In this paper an attempt has been made to establish the extent to which various barbiturates can depress conduction through mammalian sympathetic ganglia and to elucidate the nature of the mechanisms underlying this action.

METHODS

Cats, premedicated with atropine sulphate (1 mg./kg.) and anaesthetized with ether followed by intravenous chloralose (80 mg./kg.), were used in most of the experiments though spinal animals were sometimes used. Arterial blood pressure was recorded from the carotid or femoral artery by a mercury manometer.

Electrical stimulation of the cervical sympathetic was by the application of square-wave pulses (usually of 0.1 msec. duration) through shielded platinum electrodes; the frequency of such pulses will be indicated for each experiment. The contractions of the nictitating membrane were recorded in the usual way.

Intra-arterial injections close to the superior cervical ganglion, and photographic recordings of action and polarization potentials, were made by the technique of Paton and Perry (1953).

The superior cervical ganglion was perfused by the methods of Kibjakow (1933) and Feldberg and Gaddum (1934); the perfusion fluid was warmed by passing the terminal part of the polythene tubing up the animal's oesophagus (MacIntosh, unpublished). The acetylcholine content of the effluent was assayed on the blood pressure of eviscerated eserinized cats under chloralose anaesthesia (for details, see MacIntosh and Perry, 1950). The perfusing fluid was Locke's solution containing eserine (1 in 200,000) and double the usual quantity of glucose (2 g./l.).

The anaesthetic potencies of several of the barbiturates were determined by Butler's method (1942). Solutions of the sodium salts of the drugs were injected into the tail veins of albino mice. The anaesthetic doses for about 35% and for about 65% of mice, respectively, were found by trial and error. Fifty mice were then used to determine anaesthetic potency—25 being given one of these doses and 25 the other. The median anaesthetic dose (AD50) was estimated by interpolation on log/probability graph paper.

Solutions of sodium salts of the barbiturates were freshly made up before each experiment. Where the sodium salt was unobtainable the acidic form was dissolved in an appropriate quantity of 0.1 N-sodium hydroxide. Amylobarbitone sodium was chosen for all qualitative work and as the standard drug for purposes of comparison in the quantitative experiments.

RESULTS

Action of Barbiturates on Ganglia

Vasomotor Ganglia

That amylobarbitone depresses vasomotor ganglia is evident from the fact that the pressor effects of nicotine are diminished by the barbiturate, while those of adrenaline are not. This is illustrated in Fig. 1. Fig. 1a shows the pressor responses induced in an atropinized spinal cat by intravenous injections, at 3 min. intervals, of 0.4 mg. nicotine acid tartrate. At the point marked by the arrow, 10 mg. amylobarbitone sodium was given intravenously in the interval between two consecutive nicotine injections. The nicotine-

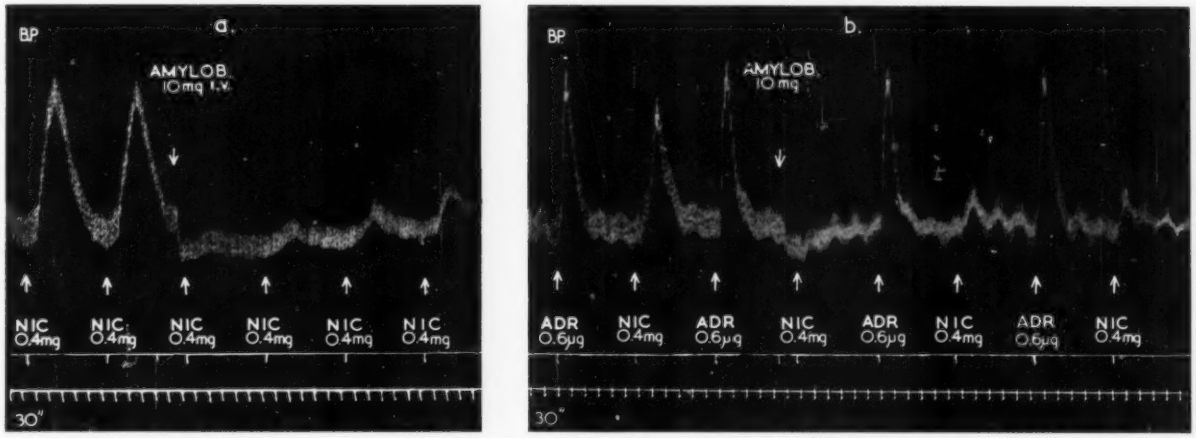

FIG. 1.—(*a*) Cat, 2.5 kg., spinal, atropinized. Blood pressure record. Pressor responses following i.v. injections of 0.4 mg. nicotine acid tartrate at 3 minute intervals. Upper arrow, 10 mg. amylobarbitone sodium i.v. (*b*) Cat, 2.7 kg., spinal, atropinized. Alternate i.v. injections of 0.6 µg. adrenaline and 0.4 mg. nicotine. Upper arrow, 10 mg. amylobarbitone sodium i.v.

induced responses were at first almost completely abolished, but later tended to recover.

That this depression of the nicotine response was not exercised on the smooth muscle of the arteriolar walls is apparent from Fig. 1b. Here, in another experiment, the pressor responses were induced by alternate injections of 0.4 mg. nicotine acid tartrate and 0.6 µg. adrenaline. Following the injection of 10 mg. amylobarbitone sodium, the nicotine pressor response was greatly reduced as before, while that of adrenaline was unaltered. Similar results were obtained with (–)-noradrenaline.

Superior Cervical Ganglion

Fig. 2 shows that amylobarbitone depressed the contraction of the nictitating membrane from electrical stimulation of the preganglionic fibres of the cervical sympathetic nerve, and that this action was exerted at the ganglion. An injection of 3 mg. tetraethylammonium iodide caused a reduction in the height of the contraction from electrical stimulation of the preganglionic fibres, but did not reduce the response to injected adrenaline. Exactly similar results followed the intravenous injection of 8 mg. amylobarbitone sodium—indicating that the site of the depressant action of the barbiturate was probably the ganglion.

During continuous preganglionic stimulation TEA intravenously produced temporary ganglion block with relaxation of the membrane, and amylobarbitone sodium intravenously gave a similar, though more prolonged, relaxation (Fig. 3a). An injection of 10 mg. amylobarbitone sodium given during postganglionic stimulation produced, however, no relaxation and, indeed, the contraction of the membrane appeared to be slightly increased (Fig. 3b). Thus amylobarbitone does not exert its effect by depressing conduction in the postganglionic nerve fibres.

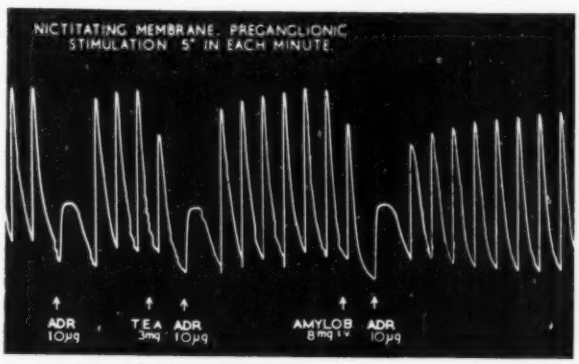

FIG. 2.—Cat, 3.0 kg., atropinized, chloralose. Nictitating membrane contractions. Periodic preganglionic stimulation (70 shocks/sec.) applied for 5 sec. in each minute. At first, third and fifth arrows the electrical stimulus was temporarily withheld and 10 µg. adrenaline given i.v. Second arrow, 3 mg. tetraethylammonium iodide i.v.; fourth arrow, 8 mg. amylobarbitone sodium i.v.

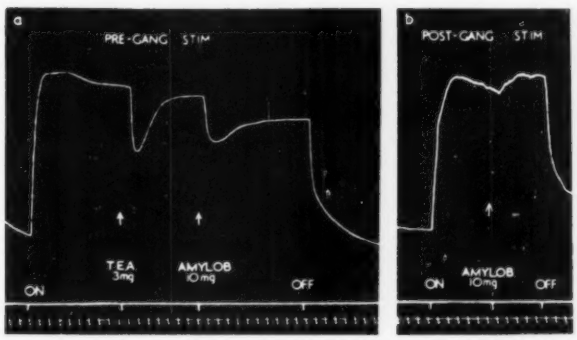

FIG. 3.—Cat, 2.6 kg., atropinized, chloralose. Nictitating membrane contraction. (*a*) Preganglionic stimulation, 10 shocks/sec. First arrow, 3 mg. tetraethylammonium iodide i.v.; second arrow, 10 mg. amylobarbitone sodium i.v. (*b*) Postganglionic stimulation, 10 shocks/sec. At arrow, amylobarbitone sodium, 10 mg. i.v.

That the preganglionic fibres are not involved in this depressant process was shown by the failure of amylobarbitone to alter or reduce the preganglionic action potentials, induced by stimulation of the preganglionic trunk low down in the neck and picked up from a point close to the lower pole of the ganglion.

It thus seems clear that amylobarbitone exerts its depressant action in, or very close to, the ganglion.

Effects of Larger Doses.—The ganglion-depressant effects of small doses of amylobarbitone appeared to be fairly transient, although cumulative effects following repeated doses became obvious by a gradual failure of the nictitating membrane contraction to return to its original level. Some experiments were accordingly done where large doses of amylobarbitone—comparable to those known to produce general anaesthesia in the cat—were used, and where the blood pressure changes were recorded. Fig. 4 illustrates such an

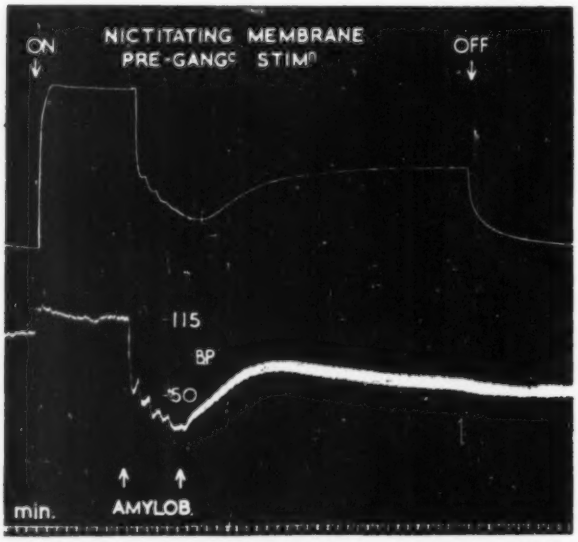

FIG. 4.—Cat, 2.5 kg., atropinized, chloralose, artificial respiration. Upper tracing, contraction of right nictitating membrane. First arrow, preganglionic stimulation commenced (10 shocks/sec.). Lower tracing, simultaneous recording of left femoral blood pressure. Lower arrow, slow i.v. injection of 50 mg./kg. amylobarbitone sodium (in 10 ml. saline). Time marker, 1 min.

experiment on an atropinized cat, anaesthetized with chloralose (70 mg./kg.) and maintained on artificial respiration to eliminate possible anoxaemia. When the blood pressure was about 115 mm. Hg, 50 mg./kg. amylobarbitone sodium—the dose recommended for intravenous anaesthesia in the cat (Garry, 1930)—was injected over a period of 5 min. This resulted in an immediate and profound reduction both in the blood pressure and in the nictitating membrane contraction. After half an hour the blood pressure had become stabilized at about 50 mm. Hg, while the contraction of the nictitating membrane had assumed a new level at about one-half its original height. Thus anaesthetic doses of amylobarbitone produce a marked and prolonged reduction in blood pressure, accompanied by a significant reduction in transmission through the sympathetic ganglion.

It is interesting to note, as was shown by similar experiments of this type, that chloralose in equivalent anaesthetic doses was almost devoid of such effects. This may well explain the particular suitability of chloralose as a laboratory anaesthetic, since it is likely to leave the efferent pathways of cardiovascular and other reflexes relatively intact.

Other Peripheral Actions of Amylobarbitone

As some earlier workers had described certain of the depressant effects of barbiturates as "atropine-like," this possibility was tested for on the cat blood pressure. The depressor effect of various doses of acetylcholine was not in the least impaired by an intravenous dose of 10 mg. amylobarbitone sodium.

Two preparations—the sciatic nerve-gastrocnemius in the spinal cat, and the isolated phrenic nerve-diaphragm of the rat—were used to determine whether amylobarbitone had any neuromuscular-blocking activity. No such activity could be demonstrated even with large doses.

Mechanism of Ganglionic Depression by Barbiturates

Depression of ganglionic transmission could result from (i) a reduction in excitability of the ganglion cells to acetylcholine, (ii) prolonged depolarization of the ganglion cells, (iii) interference with the release of acetylcholine by the preganglionic terminals, or (iv) a combination of two or more of these factors.

Excitability of Ganglion Cells.—The effect of amylobarbitone on the contractions of the nictitating membrane evoked by stimulation of the ganglion by intra-arterial injections of acetylcholine and potassium chloride was determined. The injections were made retrogradely into the cut stump of the external carotid artery (Paton and Perry, 1953) and, in Fig. 5, 4 mg. potassium chloride and 100 μg. acetylcholine were given alternately, each in 0.2 ml. isotonic saline. At the upper arrow 20 mg. amylobarbitone sodium was injected into the *femoral vein.* Responses of the nictitating membrane to acetylcholine were reduced, but those to potassium were augmented.

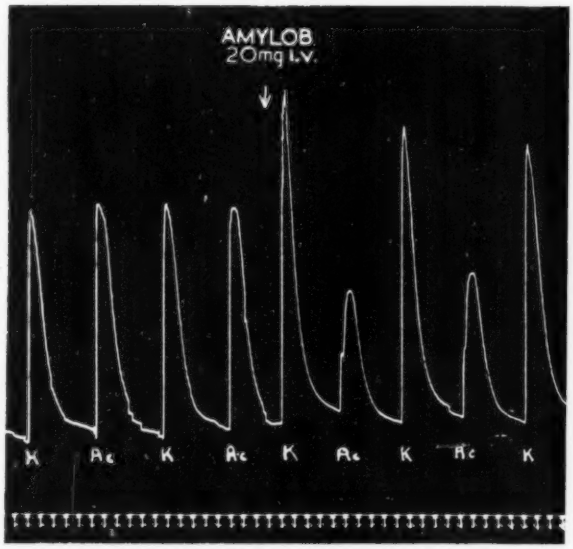

FIG. 5.—Cat. 3.8 kg., atropinized, chloralose. Contractions of the nictitating membrane. Retrograde injections into the cannulated external carotid artery of 4 mg. potassium chloride (K) and 100 μg. acetylcholine chloride (Ac), respectively. Upper arrow, 20 mg. amylobarbitone sodium i.v. Time in $\frac{1}{2}$ min.

Thus, amylobarbitone acts, in part at least, by reducing the excitability of the ganglion cells to acetylcholine. The direct stimulant action of potassium ions is not similarly impaired and is, indeed, increased—perhaps by a temporary shift in the sodium-potassium balance following the sudden introduction of sodium ions with the injected barbiturate.

Polarization Changes in the Ganglion.—Since Paton and Perry (1953) have shown that certain ganglion-blocking agents produce maximal, or near maximal, depolarization of the ganglion cells, the possibility that amylobarbitone did so too was investigated. Fig. 6 shows four oscillograms from an experiment in which the technique of these workers was used.

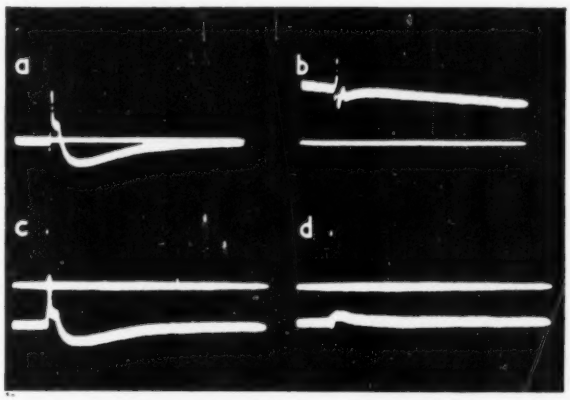

FIG. 6.—Oscillograms of ganglionic action potentials. Horizontal undeflected beam represents arbitrary reference line. Approximate duration of scan, 500 msec. (*a*) Normal action-potentials evoked by single preganglionic shock. (*b*) Effect of 100 μg. acetylcholine injected intra-arterially. (*c*) Record of normal potentials, showing stimulus artifact. (*d*) Effect of 2 mg. amylobarbitone injected intra-arterially.

In each instance the reference line was produced by one beam of the cathode-ray tube which was deflected by the horizontal time-base only. The other beam displayed amplified potentials picked up between two electrodes, one placed on the lower pole of the body of the ganglion and the other on the cut postganglionic trunk ; the latter electrodes served as the " indifferent " electrode. Negativity of the ganglion with respect to the cut postganglionic trunk is represented by upward deflections of the beam.

Each oscillogram shows the brief action-potential complex initiated by a single shock applied to the preganglionic trunk. A depolarizing substance such as acetylcholine causes the whole beam to be shifted in a negative direction while the action potential complex from the preganglionic stimulus is partially suppressed (Fig. 6b). After the intra-arterial injection of 2 mg. amylobarbitone sodium, the negative spike potential and after-positivity are almost abolished (Fig. 6d), but there is no significant change in the polarization of the ganglion. Thus the ganglion-depressant action of the barbiturate is not contributed to by depolarization phenomena.

Release of Acetylcholine.—The superior cervical ganglion was perfused with eserinized Locke solution and the preganglionic trunk stimulated continuously at 10 shocks/sec. At a predetermined time after the commencement of stimulation a dose of 50 mg./kg. of amylobarbitone sodium in eserinized Locke was injected into the perfusion tubing. Samples of the effluent from the ganglion were collected at two-minute intervals and assayed for total acetylcholine content. The amylobarbitone in the effluent did not inactivate the acetylcholine nor did it exert " atropine-like " effects during the assay. Fig. 7 shows the results from a typical experiment. Amylobarbitone produced a marked interference with ganglionic transmission, but the graph of acetylcholine output was indistinguishable from that obtained in the absence of amylobarbitone.

It is concluded that anaesthetic concentrations of amylobarbitone produce marked impairment of transmission in the perfused ganglion but that this is not contributed to by an interference with acetylcholine release. In other experiments, however, high concentrations of amylobarbitone, exceeding the anaesthetic level by some eight times, did cause an interference with acetylcholine release.

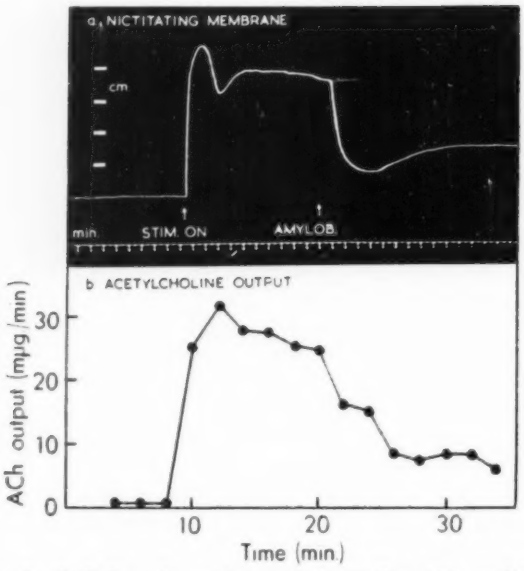

FIG. 7.—Perfusion of superior cervical ganglion with eserinized Locke solution. Upper part (*a*), record of nictitating membrane contraction. Continuous preganglionic stimulation from first arrow. Second arrow, 0.5 mg. amylobarbitone sodium in 10 ml. Locke injected into perfusion tubing. Time marker, 1 min. Lower part (*b*), graph of corresponding output of acetylcholine (mμg./min.) from ganglion, on same horizontal time scale.

Quantitative Comparison of Barbiturates as Ganglionic Depressants

Quantitative differences in the potency of the barbiturates on the ganglion are, of course, to be expected on the grounds of their varying potencies as central depressants. Whether their potencies as ganglionic depressants could be directly correlated with their C.N.S. potencies was of considerable interest.

Pentobarbitone and amylobarbitone were chosen for preliminary quantitative comparisons, since, though these drugs are isomeric, they have different potencies as C.N.S. depressants, amylobarbitone possessing about two-thirds the potency of pentobarbitone as a hypnotic in animals.

Comparisons on Vasomotor Ganglia

Preliminary experiments consisted in comparing the effects of barbiturates on the pressor responses induced in the spinal, atropinized, cat by periodic injections of choline *m*-bromophenyl ether bromide. This substance was shown by Hey (1952) to possess a nicotine-like stimulant activity on ganglia at least 10 times greater than that of nicotine itself, but with little tendency to produce secondary nicotine-like paralysis. It is thus a very suitable agent for inducing repeated pressor responses by ganglionic stimulation.

Fig. 8 shows part of a recording of the arterial blood pressure taken from such an experimental comparison. Each signal mark indicates the point of injection of 10 μg. of the choline ether into the femoral vein, resulting in pressor responses at approximately 3 min. intervals. At the first arrow, 5 mg. pentobarbitone sodium was injected intravenously ; this resulted in a reduction in the height of the succeeding pressor responses by about one-third, with gradual recovery on subsequent injections of the nicotine-stimulant drug. At the second arrow an injection of 5 mg. amylobarbitone sodium caused a much more marked reduction, with slower recovery. Thus, from this experiment, it appeared that pentobarbitone, though a stronger hypnotic than amylobarbitone, was a weaker ganglionic depressant.

Comparisons on Superior Cervical Ganglion

Factors Affecting Sensitivity of Ganglion to Drugs.—Potency tests of drugs producing block of the superior cervical ganglion are most commonly made by comparing their actions in relaxing the contraction of the nictitating membrane during continuous preganglionic stimulation at frequencies of around 10 shocks per second (e.g., Paton and Zaimis, 1951 ; Wien, Mason, Edge, and Langston, 1952). This method is not, however, very suitable for comparisons of the barbiturates: these produce cumulative effects during an experiment, so that relaxations of the nictitating membrane may not be followed by complete restoration of the contraction to its original level. A method was therefore evolved in which periodic stimulation of the preganglionic trunk was

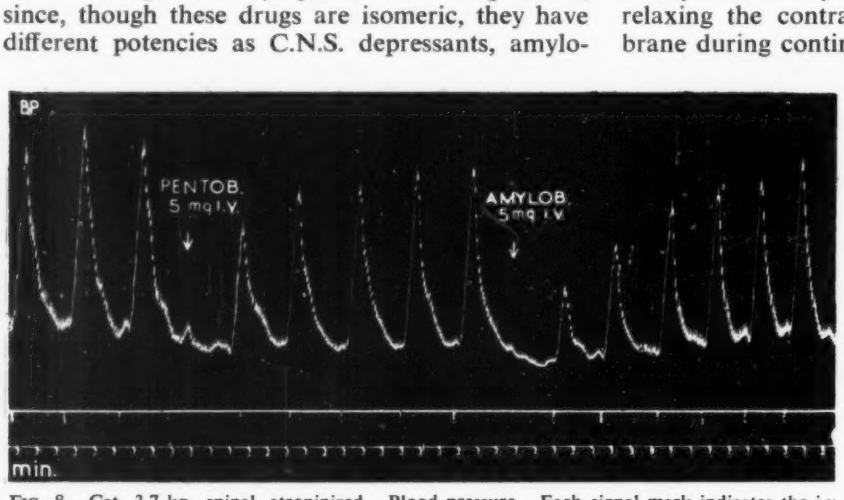

FIG. 8.—Cat, 3.7 kg., spinal, atropinized. Blood pressure. Each signal mark indicates the i.v. injection of 10 μg. choline *m*-bromophenyl ether bromide. First arrow, 5 mg. pentobarbitone sodium i.v. Second arrow, 5 mg. amylobarbitone sodium i.v.

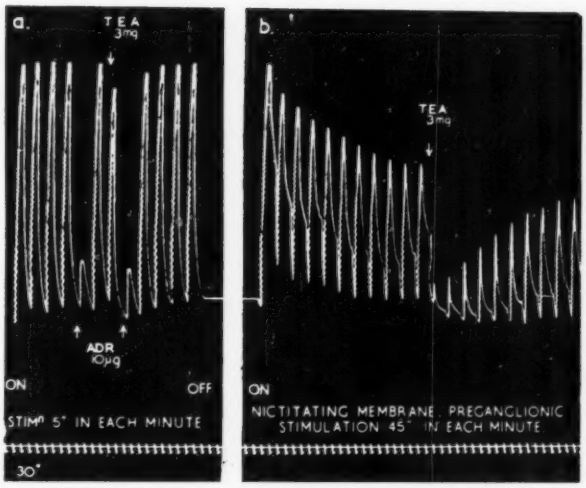

FIG. 9.—Cat, 2.6 kg., atropinized, chloralose. Contractions of nictitating membrane in response to preganglionic stimulation (70 shocks/sec.). (*a*) Stimulation for 5 sec. in each minute. Lower arrows, injections of 10 μg. adrenaline i.v. Upper arrow, 3 mg. tetraethylammonium iodide i.v. (*b*) Stimulation for 45 sec. in each minute. Upper arrow, 3 mg. tetraethylammonium iodide i.v.

used and the *proportionate* reduction in the contraction-height of the membrane following a dose of barbiturate gave a measure of the response. Absolute heights of contraction, and fluctuations of the base-line during experiments, are thus less important.

Compared with hexamethonium, or even tetraethylammonium, barbiturates are relatively weak ganglionic depressants. In attempting to assess barbiturate effects a sensitive method was therefore required. The sensitivity of the superior cervical ganglion to blocking agents can be greatly influenced both by the duration and frequency of a tetanic stimulus. The enhancement of sensitivity following increased frequency of stimulation has been remarked upon by Paton and Zaimis (1951), and the use of frequencies up to 50 shocks per second has recently been advocated (Morrison and Paton, 1953). In the present work frequencies of 50 or 70 per second were used.

The *duration* of the periods of tetanus also influences the sensitivity to a blocking agent, as shown in Fig. 9. When the stimulus is applied for 5 sec. in each minute TEA has a much smaller effect than when the stimulus is applied for 45 sec. in each minute. Stimulation for 45 sec. in each minute thus renders the preparation very responsive to blocking agents, and is therefore the most suitable for comparing weak ganglionic depressants. Its use for comparing amylobarbitone sodium and TEA iodide is shown in Fig. 10. This record clearly shows the reduction in height of the contractions attributable to several injections of the two substances. In each instance the drugs were injected 30 sec. after beginning a period of stimulation (and, therefore, 15 sec. before the stimulus ended), and the measure of response was the reduction in height of the ensuing contraction expressed as a percentage of the previous contraction. The results of this experiment are shown graphically in Fig. 11, which indicates that the two dose-response curves are reasonably linear and parallel. The activity of amylobarbitone sodium works out at a little under one-quarter that of TEA iodide, on either a weight-for-weight or a molar basis.

Comparison of Fifteen Barbiturates with Amylobarbitone.—The same method has been applied to some fifteen of the commoner barbiturates using amylobarbitone sodium throughout as the standard drug. It was usually possible to compare three or four drugs on the same animal, and wherever possible at least two doses of the standard and of the unknown were given. With certain drugs, such as the thiobarbiturates, which exhibit very weak ganglionic effects, but powerful central effects, it was only feasible to administer one dose and to match this against two or more doses of the standard. Otherwise, the total dose of thiobarbiturate would have exceeded the full anaesthetic dose before much useful information could be gained.

The dose-response curves obtained in each experiment appeared to be reasonably parallel and

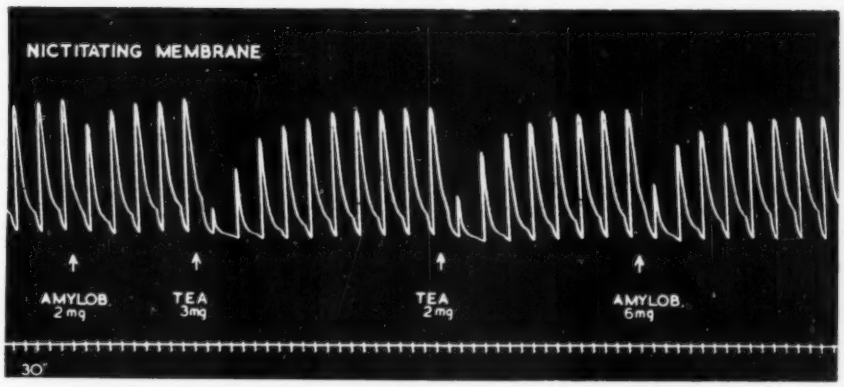

FIG. 10.—Cat, 2.7 kg., atropinized, chloralose. Recording of contractions of the nictitating membrane evoked by periodic stimulation of the preganglionic trunk for 45 sec. in each minute (as in Fig. 9*b*). The four arrows indicate, respectively, intravenous injections of 2 mg. amylobarbitone sodium, 3 mg. and 2 mg. tetraethylammonium iodide, and 6 mg. amylobarbitone sodium.

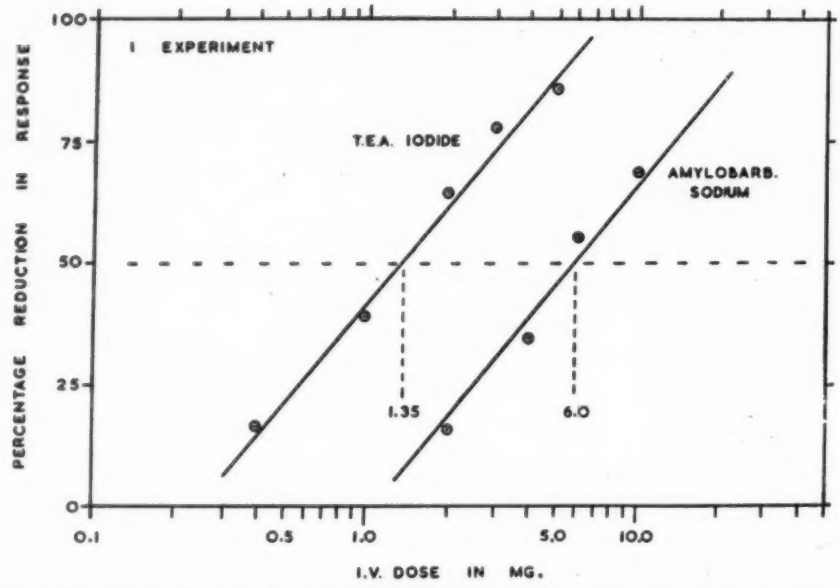

FIG. 11.—Graph of results from the experimental comparison (Fig. 10) of the ganglion depressant activities of tetraethylammonium iodide and amylobarbitone sodium. Ordinate, percentage reduction of nictitating membrane contraction. Abscissa, dose (mg.) in logarithmic scale.

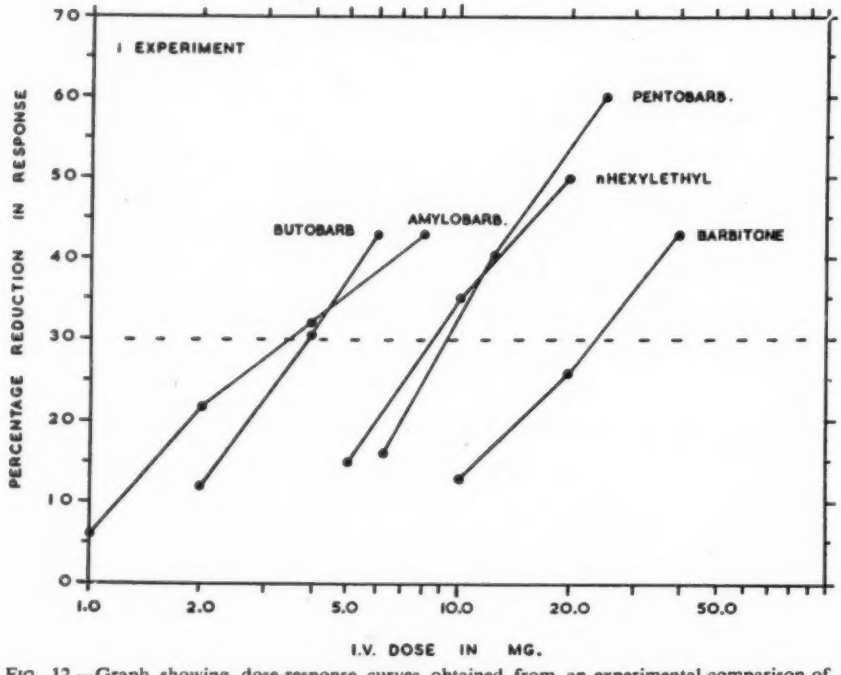

FIG. 12.—Graph showing dose-response curves obtained from an experimental comparison of the ganglion-depressant activities of the sodium salts of amylobarbitone, butobarbitone, pentobarbitone, barbitone, and *n*-hexyl-ethyl barbituric acid. Abscissa and ordinate as in Fig. 11.

activity ratios were assessed from them by graphical methods. Examples of typical dose-response curves obtained from a comparison, in the same animal, of amylobarbitone, butobarbitone, pentobarbitone, 5-*n*-hexyl-5-ethyl barbituric acid, and barbitone are shown in Fig. 12. Relative to amylobarbitone, butobarbitone is equiactive on the ganglion, pentobarbitone and the *n*-hexyl derivative are a little over one-third as active, and barbitone about one-fifth.

The results of such comparisons of fifteen barbiturates with amylobarbitone are presented in Table I, column A. Certain of these results have already been published (Exley, 1952). In each instance the potency on the ganglion is expressed, on a weight-for-weight basis, in terms of amylobarbitone = 100.0. Amylobarbitone and butobarbitone are the most active ganglionic depressants. Pentobarbitone, which is a more potent anaesthetic agent than amylobarbitone, has 42% of its activity on the ganglion—a figure which, as revealed by the standard error, differs significantly from that of amylobarbitone. Barbitone and phenobarbitone show relatively low activities of 17 and 14 respectively, while thiopentone and thialbarbitone come low down the list with activities of about 13 and 7 respectively.

Relationship of Ganglionic to Central Effects

It was of interest to compare figures for relative activities on the ganglion with those relating to C.N.S. depression. For this purpose the median anaesthetic doses as determined intravenously on mice by Butler (1942) were used. From Butler's data relative anaesthetic potencies of the drugs listed in Table I have been calculated in terms of amylobarbitone = 100.0, and are shown in column B. (The intravenous anaesthetic dose for thialbarbitone was not given by Butler and so was determined by the writer.)

TABLE I

RELATIVE GANGLION-DEPRESSANT AND INTRAVENOUS ANAESTHETIC POTENCIES OF THE COMMONER BARBITURATES

Except for thialbarbitone, anaesthetic potencies given in this table were calculated from data of Butler (1942).

Barbiturate	No. of Expts.	A Mean Potency on Sup. Cerv. Gang. ±S.E.	B Relative Anaesth. Potency	Ratio A/B
Amylobarbitone		100·0	100	1·00
Butobarbitone	8	99·1±6·2	71	1·40
n-Hexylethyl barbituric acid	6	43·4±2·7	69	0·63
Pentobarbitone	7	42·5±1·5	164	0·26
*iso*Propylallyl barbituric acid	3	32·8±3·1	93	0·35
Hexobarbitone	3	31·7±2·9	186	0·17
*iso*Butylallyl barbituric acid	3	31·6±3·8	86	0·37
Cyclobarbitone	3	31·0±5·7	49	0·63
Methylphenobarbitone	3	28·0±3·6	108	0·26
Quinalbarbitone	3	27·9±1·5	193	0·15
Barbitone	6	17·1±1·3	23	0·74
*iso*Propylethyl barbituric acid	3	16·2±1·1	49	0·33
Allobarbitone	3	15·7±0·8	74	0·21
Phenobarbitone	4	14·2±0·6	40	0·35
Thiopentone	3	12·8±1·2	450	0·03
Thialbarbitone	3	7·1±0·4	146	0·05

It is interesting to note, from the figures given in columns A and B for the different drugs, that ganglionic and anaesthetic potencies appear to show no direct correlation one with the other. The relationship of ganglionic to central nervous depressant activities is expressed in the fifth column of Table I as the ratio A/B. Such treatment of the data has the added advantage of cancelling out molar differences. The highest A/B ratio is given by butobarbitone (1.40), and the lowest by thiopentone (0.03).

All these determinations of the activity of the barbiturates were made after intravenous injections, and it was considered possible that some of the observed differences might be explicable by physical factors—such as the rate of distribution of the various drugs within the body tissues. However, when the drugs were injected directly into the arterial blood supply of the ganglion, the order of their relative ganglion-depressant potency was unchanged.

Investigation of Homologous Series of Barbiturates

Inspection of the figures for the ratio A/B, in Table I, affords very little indication of the type of chemical structure to be associated with either increased or decreased ganglionic as compared with central depressant activity. In general, it is apparent that higher values for the ratio are given by drugs such as butobarbitone, barbitone and the *n*-hexyl derivative, which contain straight alkyl chains substituted in position-5 of the barbituric acid ring. Derivatives with branched alkyl chains or complex groupings in position-5, or incorporating structural modifications of the barbituric acid ring, tend to exhibit lower values for the ratio.

It was therefore thought that an investigation of homologous series of primary alkyl-ethyl and primary di-alkyl barbituric acids might yield information on the relationships between structure and ganglion-depressant activity.

Data for certain members of these two series—barbitone, butobarbitone and *n*-hexyl-ethyl barbituric acid—are already available from Table I.

Other barbiturates containing the following substituents in position-5 were specially synthesized for this investigation:

n-propyl-ethyl ; *n*-amyl-ethyl ;
di-*n*-propyl ; di-*n*-butyl ; di-*n*-amyl.

These derivatives were compared with amylobarbitone on the superior cervical ganglion of the cat by the method previously described. The median anaesthetic dose for each was also determined on mice, by Butler's method. Anaesthesia by the di-*n*-butyl and di-*n*-amyl derivatives occurred immediately after injection into the tail vein, and was of very short duration. The figure obtained for the di-*n*-amyl derivative was less accurate because of this short duration (less than three minutes) and because the drug showed convulsant tendencies with doses near to the median. These two substances obviously possess low therapeutic coefficients, since, with doses but slightly in excess of the AD50, the animals commonly died.

Results of these tests are presented in Table II and, together with those for barbitone, butobarbitone and *n*-hexyl-ethyl barbituric acid (Table I), are shown graphically in Fig. 13. Whilst relative ganglionic and anaesthetic potencies tend to run roughly parallel for the alkyl-ethyl series—reaching a peak at the *n*-amyl-ethyl member, in the

TABLE II

RELATIVE GANGLION-DEPRESSANT AND INTRAVENOUS ANAESTHETIC POTENCIES OF CERTAIN ALKYL-ETHYL AND DI-ALKYL BARBITURIC ACIDS

Substituent in Position-5 of Barbituric Acid	A Mean of Three Potency Determinations on Sup. Cerv. Gang. ±S.E.	AD50 (mg./kg.)	B Relative Anaesth. Potency	Ratio A/B
*iso*Amyl-ethyl (amylobarbitone)	100·0	52	100	1·00
n-Propyl-ethyl	33·1±2·8	126	41	0·81
n-Amyl-ethyl	125·7±1·1	47	111	1·13
di-*n*-Propyl	48·2±3·6	136	38	1·27
di-*n*-Butyl	112·8±1·7	45	116	0·97
di-*n*-Amyl	16·4±2·8	c25	c208	c0·08

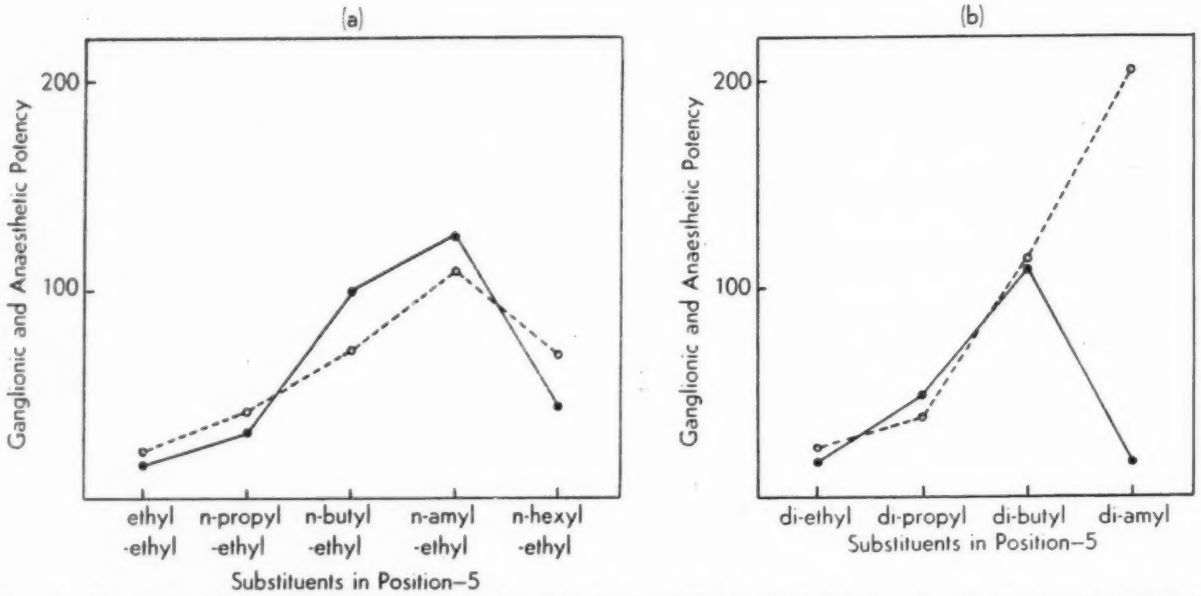

FIG. 13.—Graphs showing properties of homologous series of (*a*) primary 5-alkyl-5-ethyl barbituric acids, and (*b*) primary 5:5-di-alkyl barbituric acids. Horizontal axes, substituent groupings in position-5. Vertical axes, ganglion-depressant and anaesthetic potency, relative to amylobarbitone = 100.0. Continuous lines, ganglion-depressant potency. Interrupted lines, anaesthetic potency.

di-alkyl series these two properties show a marked divergence at the di-amyl member.

It is of interest to note that, in both series, the highest values for the ratio A/B are given by those members containing a substituent with a total of six carbon atoms—*n*-butyl-ethyl and di-propyl, respectively. A very low value for this ratio is given by the di-amyl member (0.08) which, in this respect, and in virtue of its high anaesthetic potency, shows some resemblance to the thiobarbiturates.

DISCUSSION

Investigation of the properties of amylobarbitone as a ganglion-paralysing agent has revealed several interesting likenesses to tetraethylammonium. Both drugs reduce the excitability of the ganglion cells to acetylcholine, but not to potassium chloride. Neither drug depolarizes the ganglion cells. Both, in ordinary doses, are devoid of atropine-like or neuromuscular-blocking activity.

The hypothesis that barbiturates prevent the release or synthesis of acetylcholine in nervous tissue (Dalton, 1950) has a particular appeal on account of its simplicity ; but the evidence afforded by studies on isolated brain tissue gives only equivocal support to it (Tobias, Lipton, and Lepinat, 1946 ; Richter and Crossland, 1949 ; McLennan and Elliott, 1951). Caution should, of course, be exercised in drawing any analogies between synapses in the central nervous system and those in sympathetic ganglia ; but, in the latter at least, amylobarbitone does not interfere with the release of acetylcholine except in concentrations far in excess of those which produce general anaesthesia in the intact animal.

Trethewie's claim (1942), that amylobarbitone diminished the release of acetylcholine from the perfused guinea-pig stomach and intestine during electrical stimulation of the vagus nerve, should be interpreted with care. The acetylcholine which he collected may have originated chiefly from postganglionic parasympathetic nerve terminals ; if the barbiturate did, in fact, cause interruption of nerve impulses at the level of *ganglionic synapses* then the output of acetylcholine from the postganglionic terminals might well have been profoundly reduced. But this does not imply that the barbiturate had affected acetylcholine release or synthesis at the vagal ganglia.

Tetraethylammonium salts are often presumed to produce ganglionic block by " receptor competition " with acetylcholine (Acheson and Pereira, 1946). It would seem unlikely that barbiturates have a similar mode of action, since their chemical structures bear little resemblance either to acetylcholine itself or to the quaternary ammonium blocking agents. On the other hand, barbitone and phenobarbitone have been shown by Fernando (1952) to block the action of acetylcholine on the heart of *Venus mercenaria*, and Welsh and Taub (1953) have since suggested that the " carbonyl "

groupings within the barbiturate molecule might form multiple hydrogen bonds with acetylcholine receptor substance.

The diminished excitability of the ganglion cells to acetylcholine, produced by amylobarbitone, might, however, be due to a less specific " blanketing " effect of the drug on the cell surface, or to a straightforward interference with essential cellular metabolic processes, such as has been described for narcotics on isolated brain tissue by Quastel and Wheatley (1932), and others. For the depression of spinal cord motor-neurone synapses by pentobarbitone, Eccles (1946) has suggested an increased stability of the nerve cell membranes to electrical changes. But, whatever the true explanation of the general phenomenon, it is felt that the term " ganglion-depressant " should be used in preference to " ganglion-blocking " in describing the effects of barbiturates upon autonomic ganglia.

It is possible that barbiturates may exert their effects on the brain by interfering with transmission across the synapses of the various internuncial neurones. The absence of similar central effects with tetraethylammonium might be indicative of fundamental differences between the mechanisms of synaptic transmission in sympathetic ganglia and in the brain, or it might derive from a failure of lipoid-insoluble quaternary salts to reach the central synapses.

The lack of a definite correlation between ganglionic and central nervous depressant potencies of the various barbiturates is difficult to explain. Ganglion-depressant potencies cannot be related, for example, to oil/water distribution coefficients of the drugs (Tabern and Shelberg, 1933); to rates of hydrolysis of the sodium salts at the *p*H of the blood (Bush, 1937); to speed of onset of anaesthesia after intravenous administration (Butler, 1942), or to the degree of binding of the drugs to blood proteins (Goldbaum and Smith, 1948). If Butler's figures can be taken as representing the relative rates of fixation of the drugs to nervous tissue, then drugs such as hexobarbitone or thiopentone, which exhibit both short lags and high anaesthetic potencies, might be expected to exhibit high ganglion-depressant potencies. However, the data of Tables I and II suggest that this is not so. Indeed, it is fairly obvious that drugs showing high ganglion-depressant activities fall into the intermediate group of barbiturates—that is, those with medium anaesthetic potencies and moderate anaesthetic lags.

A further possibility is that the variations in ganglion-depressant activity seen after intravenous injection might be influenced by differences in the rate at which the drugs pass from the blood stream into certain body tissues. However, whilst Brodie and his co-workers (1950, 1952) have shown that thiopentone is rapidly taken up by the fat depots of the body, thereby accounting for the short duration of action of the drug, they were unable (1953) to demonstrate a similar effect with pentobarbitone—which tends to remain equally distributed throughout the tissues. Thus variation from differences in distribution would seem to apply only to the thiobarbiturates. Further evidence that distribution differences do not contribute to the variations in ganglion-depressant potency is afforded by the fact that barbiturates injected directly into the blood supply of the ganglion exert their effects before entering the general circulation, yet they still show differences in activity similar to those found after intravenous administration.

From a consideration of the various possibilities outlined above we are forced to conclude that stereo-chemical rather than physico-chemical factors are primarily responsible for variations in ganglion-depressant activity among the barbiturates.

An important clinical implication of the results presented in Table I relates to surgical anaesthesia. It will be noted that the ratios of ganglionic to anaesthetic potency are low for the three commonly used intravenous anaesthetic agents hexobarbitone, thiopentone and thialbarbitone. Indeed, the ratio for thiopentone (0.03) is the lowest recorded. Low ratios such as these indicate low autonomic depressant activity so that the fall in blood pressure occurring during induction of anaesthesia with these drugs would be expected to be trivial. Clinical experience confirms this expectation; the fall in blood pressure with thiopentone is rarely serious and is usually less than that with intravenous hexobarbitone.

In contradistinction, drugs like amylobarbitone and butobarbitone, which show high A/B ratios, would be expected to produce more marked autonomic depression during induction of anaesthesia. Again, clinical experience confirms that serious falls in blood pressure, sometimes amounting to circulatory collapse, may occur with intravenous amylobarbitone sodium (Zerfas, 1930). It is suggested, therefore, that the routine laboratory testing of a new barbiturate, proposed for use as an intravenous anaesthetic, might profitably include an assessment of its ganglion-depressant potency.

A further practical point arising from this work concerns the choice of suitable barbiturates for the sedative treatment of conditions such as hyper-

tension and peptic ulceration, where depression of autonomic activity seems to be particularly desirable. On the basis of the results reported here, it is possible that, in this respect, butobarbitone and amylobarbitone might offer slight advantages over other members of the series. But the lack of any clearly defined structural requirements for autonomic depressant activity, as compared with central depressant activity, offers little help in the synthesis of new barbiturates with greater ganglion-depressant activities.

Finally, it is clear from these animal experiments that depression of sympathetic ganglia may play some part in contributing to the lowering of arterial blood pressure in patients subjected to the so-called "Sodium Amytal Sedation Test." The observations of Cady, Horton, and Adson (1936), that amylobarbitone is more effective than intravenous thiopentone in such a test, is now explicable on the basis of the respective ganglion-depressant activities of the two drugs.

Summary

1. Small intravenous doses of amylobarbitone sodium produce transient depression of sympathetic ganglia in cats. This is demonstrated both upon vasomotor ganglia stimulated by nicotine, and upon the superior cervical ganglion stimulated electrically.

2. The site of depression is the ganglionic synapse: neither the preganglionic and postganglionic nerve trunks nor the effector organs are involved.

3. Amylobarbitone, like tetraethylammonium, reduces the excitability of the ganglion cells to acetylcholine but not to potassium. It does not depolarize the ganglion, nor does it interfere with acetylcholine-release.

4. Intravenous anaesthetic doses of amylobarbitone cause a marked and prolonged fall in arterial blood pressure accompanied by simultaneous impairment of transmission through the superior cervical ganglion.

5. In doses which produce easily demonstrable ganglionic depression, amylobarbitone shows neither atropine-like nor neuromuscular-blocking properties.

6. A sensitive method is described for the comparison of weak ganglion-depressant agents in cats. The method involves periodic, instead of continuous, stimulation of the cervical sympathetic nerve and permits the determination of dose-response relationships for several drugs in the same animal.

7. Comparisons of amylobarbitone sodium and tetraethylammonium iodide as ganglion-depressants show that amylobarbitone has about one-quarter the potency of tetraethylammonium.

8. The ganglion-depressant activities of 15 of the commoner barbiturates were compared with that of amylobarbitone. The most active drugs are butobarbitone and amylobarbitone; the least active are the thiobarbiturates. Homologous series of primary alkyl-ethyl and di-alkyl barbituric acids were similarly examined.

9. Quantitative differences in ganglion-depressant activity are still demonstrable when the drugs are injected directly into the arterial supply to the ganglion.

10. Ganglion-depressant potencies show little correlation with central nervous depressant potencies. Possible explanations for the variability of ganglion-depressant activity are discussed.

11. It is argued that the fall in blood pressure which may follow administration of barbiturates to man is contributed to by a depression of the vasomotor ganglia. This is particularly likely to be so in people subjected to the "Sodium Amytal Sedation Test."

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THE EFFECT OF A SPECIFIC ANTISERUM ON THE ACTIVITIES OF LACTIC DEHYDROGENASE OF MAMMALIAN MUSCLE AND OF *SCHISTOSOMA MANSONI**

BY

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In a previous study the reaction kinetics of lactic dehydrogenase of *Schistosoma mansoni* were compared with those of lactic dehydrogenase of rabbit muscle (Mansour and Bueding, 1953). Our observations suggested that the enzyme of the parasite may not be identical with that of the mammalian host although both enzymes catalyse the same reaction. More conclusive evidence about this problem could be obtained by the use of an antibody against either one of these enzymes. If the latter are not identical, differences in their interaction with such antibodies would reveal differences in their nature. On the other hand, if the two enzymes are identical then they should exhibit similar behaviour toward specific antibodies.

After repeated injections of rabbit muscle lactic dehydrogenase into roosters their sera inhibited the activity of this enzyme. These sera did not affect the activity of lactic dehydrogenase of *Schistosoma mansoni* and of *S. japonicum*. However, they did inhibit the activity of rat muscle lactic dehydrogenase. In addition, information was obtained about the mechanism by which these antisera inhibit enzymatic activity.

METHODS

Enzyme Preparations.—Lactic dehydrogenase of rat muscle was purified by an adaptation of the method used by Kornberg and Pricer (1951) for the enzyme of rabbit muscle. After decapitation the rat was skinned and the carcass placed in cracked ice. All subsequent operations were carried out at temperatures varying between 0 and 4° C. The muscles were excised, passed through a meat grinder and extracted twice with 2 volumes of 0.03N-KOH for 10 minutes with occasional stirring. The extract was separated from the insoluble portion by straining through cheese cloth. The combined extracts, when assayed according to Kornberg and Pricer (1951), contained 37.5 units per ml. and 3.6 units per mg. of protein. To 154 ml. of this extract (obtained from 86 g. of muscles) was added 175 ml. of a solution of ammonium sulphate (saturated at room temperature; adjusted to *p*H 7.6). The resulting precipitate was centrifuged and dissolved in distilled water (77 ml.; 125 u./ml. and 5.2 u./mg. of protein). To 72 ml. of this fraction 27.3 ml. of the saturated ammonium sulphate solution was added and the mixture was centrifuged. To the supernatant fluid (72 ml.) 30 ml. of the saturated ammonium sulphate solution was added, the resulting precipitate was centrifuged and dissolved in distilled water (22 ml.; 330 u./ml., 20 u./mg. of protein). A solution of ammonium sulphate saturated at 2° C. (*p*H not adjusted) was added gradually in small portions until the first appearance of a turbidity. The mixture was then allowed to stand at room temperature for 10 minutes. The precipitate was centrifuged and dissolved in cold water (9 ml.; 500 u./ml., 75 u./mg. of protein). To 8.5 ml. of this solution 34 ml. of cold distilled water and 8.5 ml. of calcium phosphate gel (dry weight 14 mg./ml.) were added. After centrifugation the residue was washed successively with 8.5 ml. of 0.05 M and 8.5 ml. of 0.15 M glycylglycine buffer (*p*H 7.5). Elution of the enzyme was carried out with 8.5 ml. of 0.5 M glycylglycine buffer (*p*H 7.5). The final eluate contained 223 u./ml. and its specific activity amounted to 223 u./mg. of protein.

Measurements of enzymatic activity and preparations of lactic dehydrogenase of rabbit muscle and of schistosomes were carried out by the same procedures as those used previously (Mansour and Bueding, 1953).

Immunological Procedures.—To three roosters, each weighing approximately 5 lb., alum-precipitated rabbit lactic dehydrogenase was administered. This was prepared as follows: To 0.6 ml. of the enzyme solution containing 10 mg. of protein 0.05 ml. of an

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alum potassium sulphate solution (1% w/v) was added. After adjustment of the *p*H tc 6.4 the total volume of the suspension was brought to 5 ml. with saline (0.9% NaCl). One course consisted in three intravenous injections of 0.5 ml. of the suspension (equivalent to 1 mg. of protein) administered every other day. Four to five days after the last injection the inhibitory effect of the serum was tested. One week thereafter another similar course was given. Four additional courses were administered after intervals each lasting between one to two weeks. To a second group of three roosters the enzyme was injected in a solution of saline instead of in an alum suspension. A single dose consisted of 1.5 mg. of protein in a volume of 0.5 ml. The schedules and courses were the same as in the first group.

Before assay the sera were heated for 30 minutes at 55° C. In addition to the inactivation of complement this procedure considerably reduced the "blank" lactic dehydrogenase activity of the serum, but produced no reduction in the inhibitory effect of sera on lactic dehydrogenase. The enzyme, diluted in 0.1 M-NaCl, was incubated with the serum for 10 minutes at room temperature. The total volume was 0.08 ml. The concentration of serum in this solution never exceeded 25% because occasionally higher concentrations of control sera (from untreated animals) had an inhibitory effect on the activity of the rabbit enzyme. At the end of the incubation period the other constituents of the reaction mixture were added. Unless stated otherwise DPN* or $DPNH_2$* were added last. The volume was 1 ml.

On a number of occasions the precipitating and agglutinating properties of anti-enzyme sera were tested. The precipitin tests were performed by the ring reaction (Boyd, 1947). Salt concentrations varying from 0.9 to 8.0% were employed for the ring reaction (Goodman, Wolfe, and Norton, 1951). The agglutination reaction was carried out by a modification of the tannic acid method of Boyden (1951) as developed by one of us (Stavitsky, 1954). Sheep red blood cells were treated with tannic acid, which modified their surface so that it reacted with proteins. Such cells were then agglutinated by the specific anti-protein sera. By this method it has been found that exceedingly small amounts of antibody and antigen can be estimated (Stavitsky, 1954). Ordinarily, the method is far more sensitive than the ring reaction. Because of the small amounts of antigen (enzyme) and antiserum available the complement fixation reaction was not employed.

* In this paper the following abbreviations are used: DPN= oxidized diphosphopyridine nucleotide; $DPNH_2$=reduced diphosphopyridine nucleotide.

Results

Sera from roosters which had received the enzyme in either form began to exhibit an inhibitory effect on the activity of rabbit lactic dehydrogenase after the third course of injections. Inhibition of enzymatic activity invariably was greater with the sera of roosters to which the alum precipitated enzyme was administered, although the dose of the enzyme was lower. After a fourth course of injections the degree of inhibition reached its maximum. This inhibitory effect remained unchanged after exhaustive dialysis of the sera against isotonic solutions of sodium chloride. Control experiments revealed that at optimal *p*H and substrate concentrations sera of untreated roosters produced no inhibitory effect when used in concentrations of 25% or less. In fact, there was a slight stimulation of enzymatic activity, presumably because of the protective action of serum proteins on the enzyme (Table I).

Incubation of the rabbit enzyme with immune sera resulted in a marked reduction of enzymatic activity. The degree of inhibition was lowered as the concentration of the serum was decreased (Fig. 1). The immune serum against the rabbit enzyme significantly affected the activity of lactic dehydrogenase of rat muscle. The inhibitory effect of equal concentrations of immune sera on the activity of the rat enzyme was about half as great as on the enzyme from rabbit muscle (Fig. 1).

TABLE I

EFFECT OF CONTROL AND OF IMMUNE SERA ON THE ACTIVITIES OF LACTIC DEHYDROGENASES FROM RABBIT AND RAT MUSCLE AND FROM SCHISTOSOMES

All activities are expressed in millimicromoles of DPN, oxidized in one minute or reduced in two minutes. E: Activity of enzyme. S: Activity of serum. Concentration of sera during pre-incubation: 25%.

Substrates	Serum	Rabbit Muscle Lactic Dehydrogenase			Rat Muscle Lactic Dehydrogenase			*Schistosoma mansoni* Lactic Dehydrogenase			*Schistosoma japonicum* Lactic Dehydrogenase		
		E+S†			E+S†			E+S†			E+S†		
		Calc.	Found	%Inhib.	Calc.	Found	%Inhib.	Calc.	Found	%Inhib.	Calc.	Found	%Inhib.
Pyruvate + $DPNH_2$	Control	15·0	16·5	0	14·4	14·8	0	15·3	16·1	0	15·6	15·8	0
	Immune	15·5	6·1	68	16·4	11·6	39	16·6	18·0	0	14·9	14·8	0
Lactate + DPN	Control	25·0	32·2	0	35·9	44·0	0	22·8	26·3	0	26·9	30	0
	Immune	24·7	13·7	56	35·6	29·0	21	23·3	26·8	0	27	29·4	0

† In these experiments the activity of the serum did not exceed 20% of that of the enzyme.

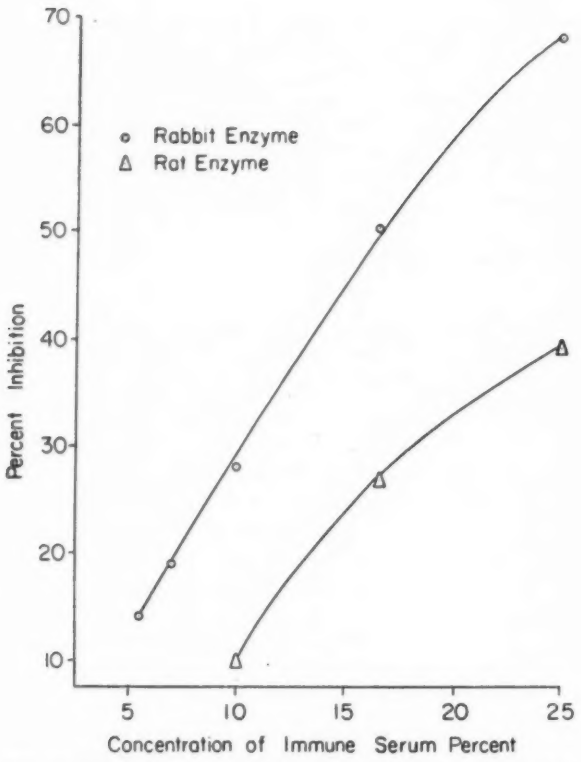

FIG. 1.—Effect of the concentration of immune serum on the activity of lactic dehydrogenase of rabbit and of rat muscle. Abscissa: concentration of immune serum %. Ordinate: % inhibition.

The sera from roosters immunized against the rabbit enzyme did not affect the activity of the lactic dehydrogenases of *Schistosoma mansoni* or of *Schistosoma japonicum* (Table I). It should be noted here that essentially identical results were obtained regardless of the direction in which the catalytic activities of the enzymes were measured —that is, whether by the reduction of pyruvate to lactate or the oxidation of lactate to pyruvate. There was an exception to this rule; the stimulatory or protective effects of normal sera on the schistosome enzymes were noticeable only during the oxidation of lactate, but not during the reduction of pyruvate.

The inhibitory effect of the immune serum on the rabbit enzyme was not altered by the presence of the lactic dehydrogenase preparation of *Schistosoma mansoni* (Table II).* Therefore, the lack of an inhibitory effect of the immune serum on the enzyme of schistosomes was not due to the destruction of the antibody by the enzyme preparation of the worms.

Incubation of the immune serum with three other glycolytic enzymes resulted in no change in their activities. The enzymes tested were phosphohexose isomerase, aldolase, and phosphoglyceraldehyde dehydrogenase. They were prepared and assayed (with and without the serum) according to Cori *et al.* (Cori, Slein, and Cori, 1948; Taylor, Green, and Cori, 1948; Slein, Cori, and Cori, 1950). Furthermore, incubation of crystalline phosphoglyceraldehyde isomerase of rabbit muscle with the immune serum did not alter the inhibitory effect of the latter on mammalian lactic dehydrogenase.

If an anti-enzyme serum inhibits enzymatic activity, it is generally assumed that this occurs by an attachment of the antibody at the site where com-

* The activity of a mixture of the two enzymes was slightly lower (approx. 10%) than the sum of the activities of each enzyme (see Table II, columns 3b and 3c). Therefore, it was necessary to make a corresponding correction in the calculation of the activity of the mammalian enzyme in this mixture (see Table II, columns 7b and 7c). Without this correction the inhibitory effect of the antiserum would have been greater in the presence of the worm enzyme than in its absence. This would appear highly improbable.

TABLE II

EFFECT OF IMMUNE SERA ON THE ACTIVITY OF LACTIC DEHYDROGENASE OF RABBIT MUSCLE IN THE PRESENCE OF A LACTIC DEHYDROGENASE PREPARATION OF *S. MANSONI*

RE=activity of rabbit enzyme, WE=activity of worm enzyme, S=activity of immune serum. Enzymatic activities are expressed in the same manner as in Table I.

Reaction	Serum	1 RE	2 WE	3 WE+RE			4 S	5 RE+S	6 WE+S	7 RE+WE+S			Inhibition %
				Found a.	Calc. b.†	Diff. c.‡				Found a.	Calc. b.§	RE c.‖	
Pyruvate→ Lactate	Immune	12·0					2·6	7·7					57
	Immune		17·2				2·6		19·7				—
	Immune	9·5	9·2	17·1	18·7	1·6	2·6			14·3	15·9	4·1	57
	Control	12·2	10·8	21·4	23·0	1·6	2·6			25	26·6	13·2	—
Lactate→ Pyruvate	Immune	22					3·4	17·5					36
	Immune		14				3·4		20·0				—
	Immune	20·0	18·0	37	38	1·0	3·6			32·5	33·5	11·9	40
	Control	22·6	13·4	35·4	36	0·6	5·6			40	40·6	21·6	4

† Sum of activities of worm enzyme and of rabbit enzyme (Columns 1 and 2).
‡ Calculated sum of activities of each enzyme alone (Column 3b) minus observed activity of mixture of both enzymes (Column 3a).
§ Observed activity (Column 7a) plus difference between calculated sum of activities of each enzyme alone minus observed activity of mixture of both enzymes (Column 3c).
‖ Calculated activity of mixture (Column 7b) minus activity of worm enzyme (Column 2) minus activity of serum (Column 4).

bination of the enzyme with the substrate or coenzyme occurs. Under these conditions the antibody would prevent the substrate (or coenzyme) from reacting with the enzyme, resulting in an inhibition of enzymatic activity. If competition for the same point of attachment to the enzyme, between antibody on the one hand, and substrate or coenzyme on the other, took place, the inhibitory effect of the antibody on the enzymatic activity might be reduced when the enzyme and its antibody are incubated in the presence of substrate or the coenzyme. Conversely, inhibition of the activity would be increased when the concentration of the competing substrate is reduced. However, pre-incubation of rabbit muscle lactic dehydrogenase with pyruvate, lactate, $DPNH_2$ or DPN did not affect the inhibitory action of the immune serum. Furthermore, a decrease in the concentration of either substrate or coenzyme resulted in a lower rather than in a higher inhibition of enymatic activity.

All attempts to demonstrate precipitation of the enzyme and agglutination of enzyme-treated red cells with the antisera were consistently negative.

Discussion

It should be noted that relatively small amounts of antigen have been employed in this study for the production of antibodies to lactic dehydrogenase. A relatively large quantity of injected soluble proteins is ordinarily degraded and lost from the body before stimulating antibody production, and it appears that the use of alum-precipitated proteins results in a more efficient utilization of the protein. On the other hand, it is known that the injection of excessive amounts of protein may lead to the phenomenon of "immunological paralysis," in which the animal fails to produce antibody (Dixon and Maurer, 1953).

The detection of antibody by the measurement of specific inhibition of enzymatic activity in the absence of visible serological activity was not expected. These observations demonstrate, at least in this instance, the superiority of the enzymatic inhibition method over serological procedures. Furthermore, unlike the serological procedures, this method does not depend on the use of highly purified antigens and antibodies because the specificity is associated with the substrate specific for the enzyme.

Kubowitz and Ott (1943) have shown that the rabbit antibody against lactic dehydrogenase of rat muscle competes with pyruvate for the active centre of the enzyme. Krebs and Najjar (1948) were able to protect phosophoglyceraldehyde dehydrogenase of rabbit muscle from inhibition by the antibody by pre-incubating the enzyme with high concentrations of DPN. Zamecnik and Lipmann (1947) found that lecithin interfered with the reaction of lecithinase of *Cl. welchii* (alpha toxin) and the antibody. In these instances the antibodies competed specifically with substrate or coenzyme for combination with the enzyme. On the other hand, there are examples where the antibody combines with the enzyme and precipitates it without inhibition of enzymatic activity (Campbell and Fourt, 1939). Furthermore, according to a hypothesis proposed by Cinader (1953), the antibody does not necessarily exert its inhibitory effect by combining with the same site on the enzyme as the substrate or coenzyme. Our observations appear to support this view because neither pyruvate nor DPN protected lactic dehydrogenase from inhibition by the antibody. Therefore, the antibody could have exerted its inhibition by combining with sites other than the active centre of the enzyme. Further study is required to provide a definitive answer regarding the nature of this combination.

In the present study it was observed that an antiserum against rabbit muscle lactic dehydrogenase inhibited the activity of this enzyme, but had no effect on lactic dehydrogenase of *Schistosoma mansoni* and of *Schistosoma japonicum*. Since inactivation or destruction of the antibody by the enzyme preparation of the worms has been ruled out it is concluded that lactic dehydrogenase of rabbit muscle is not identical with lactic dehydrogenase of schistosomes although the enzymes catalyse the same reactions. This conclusion is supported also by differences in the kinetics of the enzymes (Mansour and Bueding, 1953). The nature of these differences remains to be determined.

The specificity of the enzyme–antibody reaction is illustrated by the observations that the immune serum did not affect the activities of three other glycolytic enzymes of rabbit muscle—phosphohexose isomerase, aldolase, and phosphoglyceraldehyde dehydrogenase. Since incubation of the last enzyme did not affect the inhibitory activity of the serum against mammalian lactic dehydrogenase it appears that the antibody reacts with specific groupings common to lactic dehydrogenase of both rabbit and rat muscle.

Further evidence that the nature of the schistosome enzyme differs from that of the host was supplied by the use of sera obtained by immunizing roosters against lactic dehydrogenase of *Schistosoma mansoni*. These sera markedly inhi-

bited the activities of lactic dehydrogenase of *Schistosoma mansoni* and of *Schistosoma japonicum* but had no effect on the rabbit or rat muscle enzymes. Moreover, in contrast to the mammalian enzyme–antibody system, pre-incubation of the enzyme with $DPNH_2$ markedly reduced the inhibition of the worm enzyme by its antibody (Henion, Mansour, and Bueding, unpublished observations) indicating that the latter may interact with the active centre of the worm enzyme. This observation and the fact that this antiserum does not affect the activity of mammalian lactic dehydrogenase suggest that the active centre of the worm enzyme differs from those of lactic dehydrogenase of rabbit and of rat muscle. The antiserum against the lactic dehydrogenase of schistosomes had no effect on the activity of phosphohexose isomerase of the same parasite; conversely, an antiserum against the latter enzyme did not inhibit the activity of lactic dehydrogenase of schistosomes (Bueding, MacKinnon, and Henion, unpublished observations). Therefore, it appears that these two antisera do not react with groupings common to all schistosome proteins but with sites specific for a particular enzyme of the parasite.

The antiserum against lactic dehydrogenase of rabbit muscle significantly inhibits the activity of lactic dehydrogenase of rat muscle, but to a lesser degree than that from rabbit muscle. The enzymes of these two mammalian species are probably similar to, but not identical with, each other.

Species differences in the nature of enzymes catalysing the same reaction might be of interest for the development of chemotherapeutic agents because they may afford opportunities to select specific inhibitors against the enzyme of the parasite without affecting that of the host.

Summary

1. Sera from roosters which had been immunized against lactic dehydrogenase from rabbit muscle markedly inhibited the activity of this enzyme but did not affect those of the lactic dehydrogenases of *Schistosoma mansoni* and of *Schistosoma japonicum*.

2. It is concluded that lactic dehydrogenase of schistosomes is not identical with that of mammalian tissues although these enzymes catalyse the same reaction in the parasite and in the host.

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THE QUANTITATIVE ASSAY OF ANGIOTONIN

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An active polypeptide known as angiotonin or hypertensin, formed by the reaction of a renal enzyme (renin) with a plasma—α2—globulin (hypertensinogen), causes vasoconstriction in perfused rabbits' ears (Page and Helmer, 1940 ; Page, 1942) and toads' hind limbs (Houssay and Taquini, 1938a, 1938b), and a rise of blood pressure in anaesthetized or pithed dogs (Braun-Ménendez, Fasciolo, Leloir, and Muñoz, 1940) and cats (Dexter, Haynes, and Bridges, 1945). Based on these effects, methods for the assay of angiotonin have been suggested, but these are not suitable for the detection and determination of the angiotonin-like activity of human plasma since they are not quantitative and are not sufficiently specific or sensitive.

A sensitive, and what is believed to be a specific, method for the quantitative assay of angiotonin has been developed and is described below. It is based on the pressor response obtained on injecting the material into the dibenamine-treated rat's blood-pressure preparation described previously by Dekanski (1952).

A comparative study of angiotonin and posterior pituitary extract with respect to both the pressor and the oxytocic actions has been made and is also reported.

METHODS

Pressor Activity of Angiotonin.—This was assayed on the blood pressure of the dibenamine-treated rat. Dibenamine effectively antagonizes the pressor responses to adrenaline, noradrenaline, isoamylamine, tyramine, nicotine and piperidine (Dekanski, 1951) but not those to pituitary extract or angiotonin. In each assay four doses were used, two of the standard and two of the unknown, the ratio of high to low dose being the same (usually 3 to 2) for both the standard and the unknown. Each dose was injected every 3 to 5 min. once in each group of four, and there were six groups in all. Solution Angiotonin (Eli Lilly, U.S.A.), 10 cat units/ml., served for both the standard and the unknown preparations. Rats weighing 250 g. or less were more sensitive to angiotonin than those weighing 300 g. and more. It was found that, in order to maintain a steady base line, the amount of dibenamine could be usefully increased to at least twice that previously suggested (Dekanski, 1952) (six repeated injections of 100 μg. dibenamine per 100 g. body weight intravenously at 5 min. intervals). When plasma was tested the ratio of high to low dose was usually 2 to 1 and there were 3 or 5 groups in all.

The pressor activity of angiotonin was also compared with that of pituitary standard (International Pituitary Posterior Lobe Standard—100 mu./ml. normal saline, freshly made each time). Each dose of pituitary standard was injected intravenously 3 to 5 min. after the preceding injection of angiotonin, and each dose of angiotonin solution was injected 6 to 8 min. after the preceding dose of pituitary. Each dose, either of pituitary standard or of angiotonin solution, was administered once in each group of four doses and there were 5 to 6 groups in all.

Oxytocic Activity of Angiotonin.—This was compared with that of pituitary standard (20 mu./ml. normal saline, freshly made each time) on the rat's uterus suspended at 30° C. in a bath containing modified Ringer–Locke solution with reduced $CaCl_2$ (60 mg./l.) and no Mg (Gaddum, Peart, and Vogt, 1949).

Inactivation of Pituitary Extract and of Angiotonin.—The inactivation of the pressor activity of pituitary extract and of angiotonin was studied by treating each of the preparations or plasma at room temperature (van Dyke, Chow, Greep and Rothen, 1942) with sodium thioglycollate, at *p*H 7.4. M-Sodium thioglycollate was freshly prepared each time from the acid with H_2O and $NaHCO_3$. The solution was added to posterior pituitary extract, angiotonin solution or plasma to give a final concentration of 0.1 M- or 0.01 M-sodium thioglycollate, and the mixture left for varying times before testing.

Action of 5-Hydroxytryptamine (Serotonin).—The action of serotonin on the blood pressure of the anaesthetized rat was studied before and after treatment with hexamethonium, or with dibenamine, or with both.

Drugs Used.—Urethane (B.D.H.), 25 g. per 100 ml. in distilled water ; dose, 0.7 ml. per 100 g. body weight, subcutaneously. Heparin B.P. (Boots) ; dose, 200 u. per 100 g. body weight, in 1 ml. normal saline,

FIG. 1.—Blood pressure of rat (246 g.). Urethane. Dibenamine 1.49 mg. Pressor responses to different doses of Solution Angiotonin (Eli Lilly)—10 cat u./ml. diluted with saline to give 2 u./ml.—ranging from 0.01 to 0.24 ml. NS, normal saline. From these results the log dose-response graph of Fig. 2 has been constructed.

FIG. 2.—Log dose-response regression line relating the responses to varying doses of angiotonin based on results shown in Fig. 1. b=44.3 mm. Hg.

FIG. 3.—Blood pressure of rat (260 g.). Urethane. Dibenamine 1.5 mg. Pressor effects of angiotonin. A, 0.06 ml., B, 0.04 ml., standard (10 u./ml.). C, 0.09 ml., D, 0.06 ml., "unknown" (7.5 u./ml.). Washed in with 0.2 ml. saline. Interval 3 min. Unknown estimated to contain 7.2 u./ml. Total experimental time 3 hr.

FIG. 4.—Blood pressure of rat (330 g.). Urethane. Dibenamine 2.31 mg. Pressor effects of angiotonin and posterior pituitary standard. A, 6 mu., B, 4 mu. posterior pituitary extract. C, 0.6, D, 0.4 "units" angiotonin. Washed in with 0.2 ml. saline. Interval after pituitary 7 min., and after angiotonin 3 min.

FIG. 5.—Uterus of oestrous rat (155 g.) in 2 ml. bath at 30° C. Comparison of oxytocic effects of angiotonin and posterior pituitary extract. A, 0.3, B, 0.2 mu., posterior pituitary. C, 0.45, D, 0.3 "units" angiotonin. Interval 4 min.

FIG. 6.—Blood pressure of rat (300 g.). Urethane. Dibenamine 1.8 mg. To show that pressor effect of posterior pituitary extract (PLS) is more prolonged than that of equipressor dose of angiotonin. Interval after angiotonin 3 min. Interval after pituitary 8 min.

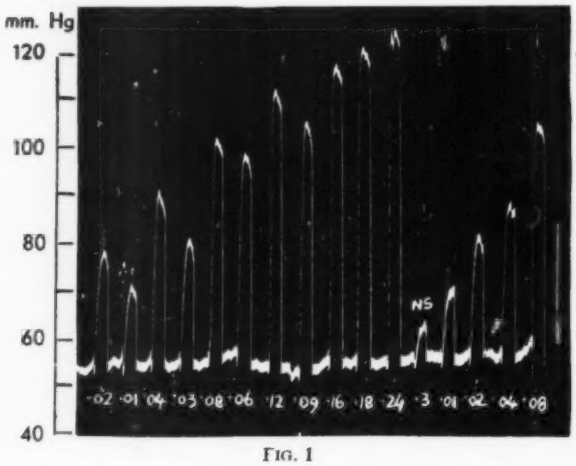

FIG. 1

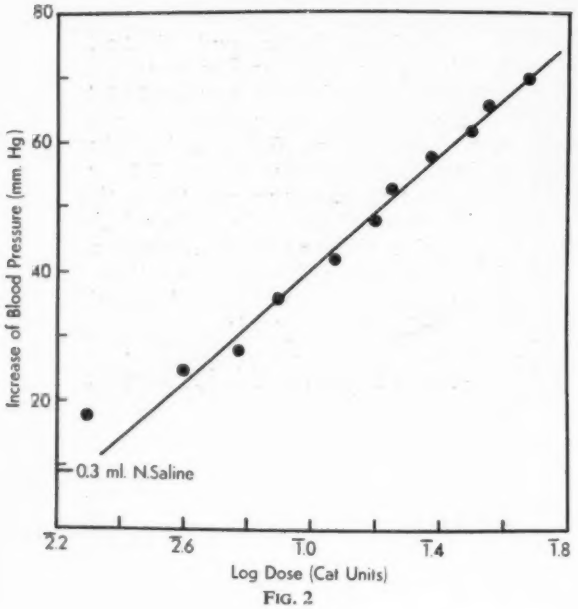

FIG. 2

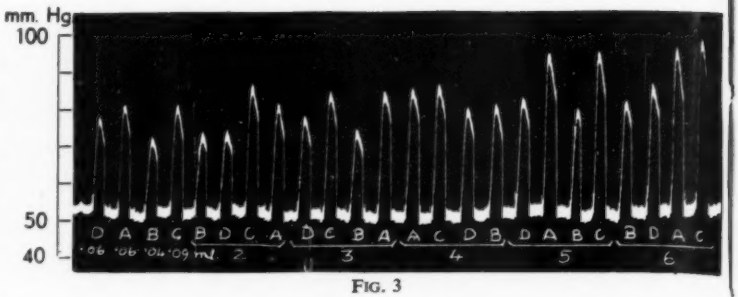

FIG. 3

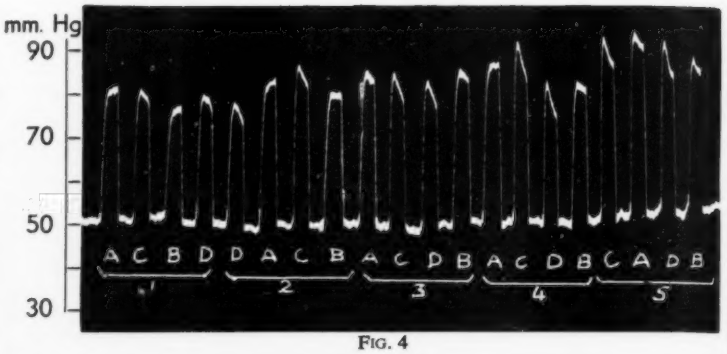

FIG. 4

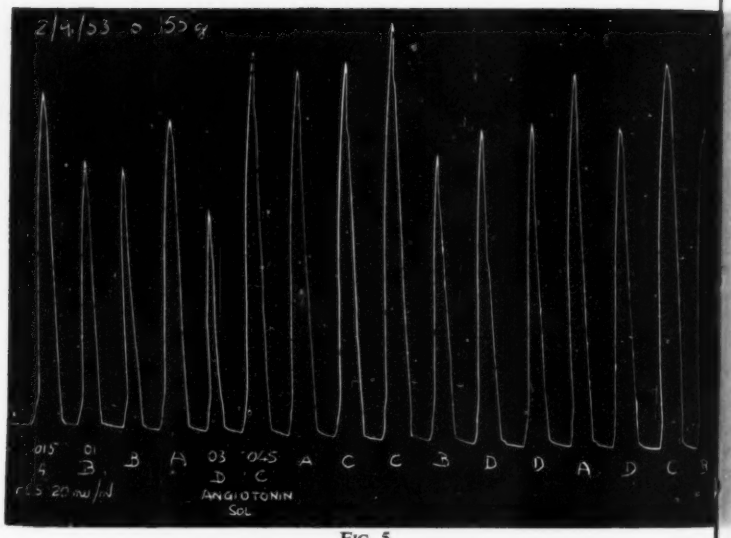

FIG. 5

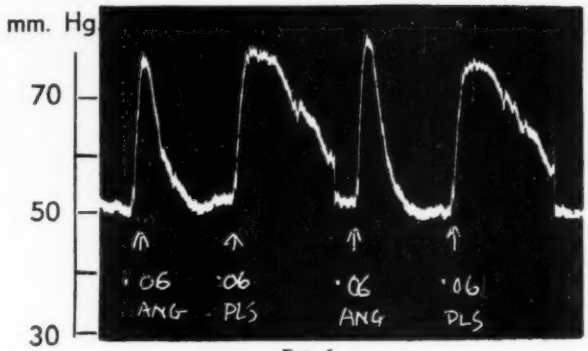

FIG. 6

intravenously. Dibenamine HCl or *NN*-dibenzyl-β-chloroethylamine (Smith, Kline, and French), 1 mg./ml. in faintly acid saline, prepared by dissolving 5 mg. dibenamine in 0.1 ml. 95% ethanol made acid (approx. 0.05 N) with conc. H_2SO_4 and then diluted up to 5 ml. with normal saline. Hexamethonium bromide, 2 mg./ml. in normal saline; dose, 1 to 2 ml. per rat, over a 10 min. period, intravenously. 5-Hydroxytryptamine creatinine sulphate, 20 μg./ml. solution in faintly acid saline (*p*H 4.5), freshly made each time from stock solution (100 μg./ml.); doses are expressed in terms of the base.

RESULTS

Quantitative Assay of Solution Angiotonin (Eli Lilly).—Fig. 1 demonstrates the blood pressure responses to increasing doses of the angiotonin solution which was used as reference standard. Fig. 2, which has been constructed from Fig. 1, shows the log dose-response regression line relating the responses (mm. Hg) to varying doses of angiotonin. A straight log-dose-response regression line could always be obtained by the method used. The preparation treated with dibenamine maintains a constant basal pressure and readily discriminates between doses differing by approximately 15%. The injections of small doses (0.02 to 0.6 cat units) can be made every 3 to 5 min. without tachyphylaxis developing, and the responses are satisfactory for about 50 injections.

The results of a pressor assay of the angiotonin are given in Fig. 3, from which data the value of M was calculated to be $\bar{1}.9693$; hence R = 0.9317. After correcting for the dose levels injected, the ratio of unknown to standard was found to be 0.72: the true value was 0.75 (0.75 ml. of standard diluted with 0.25 ml. normal saline). The potency of the unknown was therefore estimated as 7.2 cat units/ml., with fiducial limits 6.64–7.70 cat units/ml. (7.4% error), instead of the known value of 7.5 cat units/ml. The slope b = 56.1 mm. Hg, and the index of precision λ = 0.036. Analysis showed that the variance due to regression was significant ($P = <0.001$), as was that due to the groups ($P = <0.001$).

Comparison of Pressor and Oxytocic Effects of Angiotonin with those of Posterior Pituitary Standard.—The results of a comparison of the pressor effect of angiotonin and of pituitary standard are given in Fig. 4. By calculation it was found that the Solution Angiotonin, 10 cat units/ml., contained pressor activity equivalent to 101.8 mu. of pituitary/ml. (approximately 1 mu. of pituitary ≡ 0.1 cat units) with fiducial limits 88.3 to 117.3 mu./ml. (14% error). The slope b = 26.1, and the index of precision λ = 0.064. Analysis of variance for deviation from parallelism showed that there was no significant difference of slope between the curves for pituitary and angiotonin (P = 0.05–0.1). Part of the results of a comparison of the oxytocic effect of angiotonin and of pituitary standard is given in Fig. 5. It was found that the Solution Angiotonin, 10 cat units/ml., contained oxytocic activity equivalent to 7.5 mu. of pituitary/ml. (approximately 1 mu. of pituitary ≡ 1.33 cat units) with fiducial limits 6.65 to 8.35 mu./ml. (11% error). The slope b = 96.9, and the index of precision λ = 0.064.

Effect of Thioglycollate (TG) on the Pressor Activity of the Posterior Pituitary, Angiotonin, and Plasma.—The pressor and oxytocic effects of the angiotonin and the pituitary standard seem to be qualitatively similar, the only difference being a rapid fall in blood pressure to the initial level after angiotonin (Fig. 6). With minute doses of angiotonin and pituitary standard the difference in responses may be undetectable. The possible occurrence of vasopressin in plasma samples or extracts makes it necessary to eliminate effects due to this substance when carrying out angiotonin assays of such samples. The differentiation between effects due to vasopressin and those due to angiotonin is readily effected by treatment of the plasma or extract with sodium thioglycollate before assaying. This treatment inactivates vasopressin but not angiotonin. Samples of plasma for these tests were obtained from patients with renal hypertension, at the onset of acute haemorrhagic nephritis. To serve as controls, several plasmas from non-nephritic and normotensive patients were

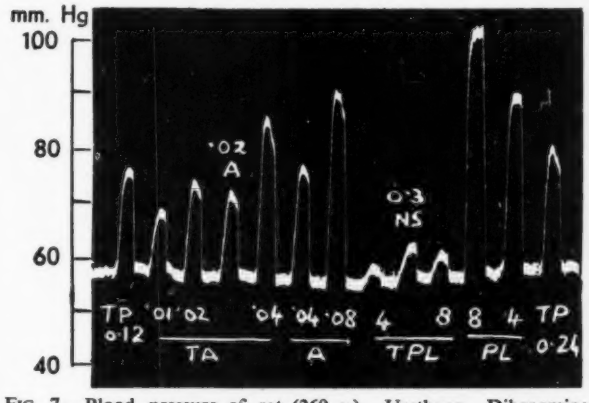

FIG. 7.—Blood pressure of rat (260 g.). Urethane. Dibenamine 2.08 mg. Pressor effects of pituitary standard (100 mu./ml.) and angiotonin standard (10 cat u./ml.) before and after 24 hr. treatment with 0.01 M-sodium thioglycollate at room temperature, and those of plasma pretreated with TG under similar conditions. TP, treated plasma; TA, treated angiotonin; TPL, treated pituitary standard; PL, pituitary standard; A, angiotonin standard; NS, normal saline. The numerals associated with the code letters indicate, for pituitary standards the dose in mu., for angiotonin the amount of standard solution in ml., and for plasma and saline the vol. in ml.

also tested. Blood was taken off into heparin and centrifuged at once; the separated plasma was chilled to 0° C. and tested as quickly as possible thereafter.

Fig. 7 demonstrates the effect of treating pituitary standard, angiotonin solution, and pathological plasma containing angiotonin-like activity, with 0.01 M-sodium thioglycollate for as long as 24 hr. The results show that TG in the stated concentration completely inactivates the pressor effects of posterior pituitary extract (100 mu./ml.) but not that of angiotonin (10 units/ml.). The results with plasma under similar conditions seem to demonstrate that the pressor action of the plasma also survives treatment with TG.

Fig. 8 provides more evidence that pituitary added to normal plasma (100 mu./ml.) has pressor activity about equal to that of pituitary standard

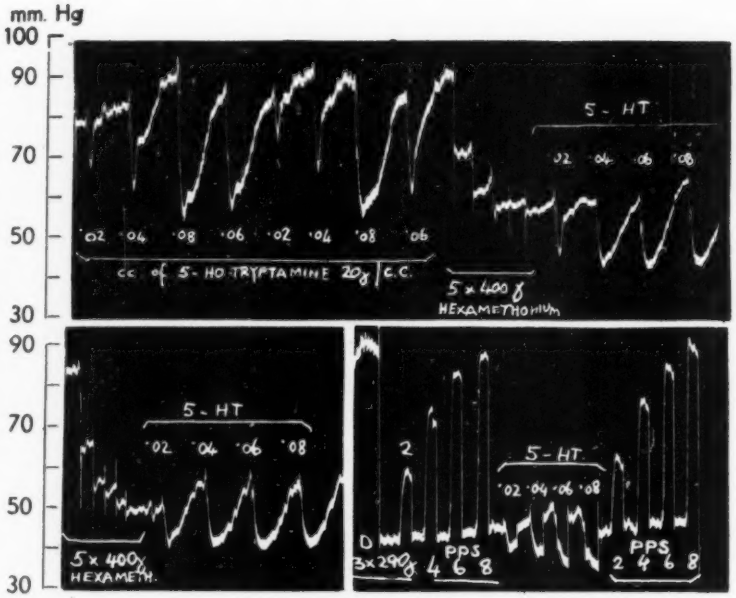

FIG. 10.—Blood pressure of rat (290 g.). Urethane. Hexamethonium 4 mg. Dibenamine 0.87 mg. Depressor effects of 5-hydroxytryptamine (0.4 to 1.6 μg.) before and after treatment with hexamethonium (twice) and dibenamine (D). PPS 2, 4, 6, and 8 mu. pituitary standard.

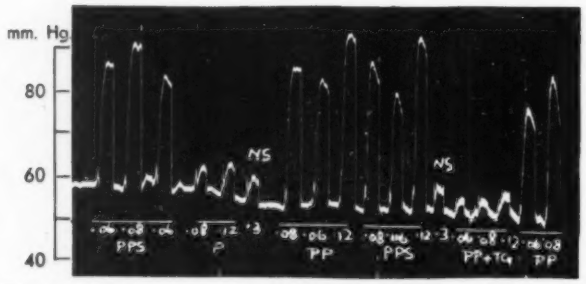

FIG. 8.—Blood pressure of rat (270 g.). Urethane. Dibenamine 1.89 mg. Pressor effects of pituitary standard (PPS 100 mu./ml.), normal plasma (P) and plasma with added pituitary standard (PP 100 mu./ml.). Note the absence of pressor responses to plasma containing added pituitary (PP 100 mu./ml.) after treatment with 0.01 M-sodium thioglycollate (PP+TG) for 40 min. at room temperature. NS, normal saline. Washed in with 0.2 ml. saline. Interval 8 min.

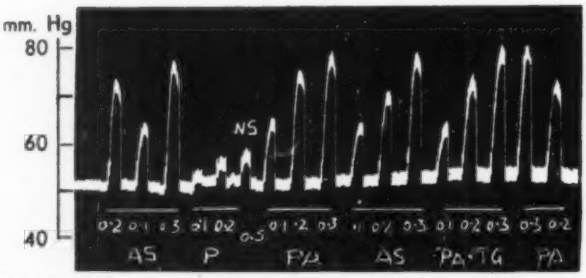

FIG. 9.—Blood pressure of rat (266 g.). Urethane. Dibenamine 1.6 mg. Pressor effects of angiotonin (AS, 2.5 u./ml.), normal plasma (P) and plasma with added angiotonin (PA 2.5 u./ml.). Note the presence of almost equipressor responses to plasma containing added angiotonin before and after treatment with 0.01 M-sodium thioglycollate (PA+TG) for 40 min. at room temperature. NS, normal saline. Washed in with 0.2 ml. saline. Interval 3 min.

(100 mu./ml.) and that the addition of 0.01 M-sodium thioglycollate to the plasma pretreated with pituitary completely inactivates its pressor effect within 40 min. On the other hand, angiotonin added to the same plasma survived the treatment with 0.01 M-sodium thioglycollate as shown in Fig. 9.

Effect of 5-Hydroxytryptamine on the Blood Pressure of the Hexamethonium-treated and Dibenamine-treated Rats.—Other pharmacologically active substances which may be present in plasma and which might interfere with the pressor assay are the vasotonins (Spätgift-Freund, 1920) released when blood is collected. It is likely that the most important of these is 5-hydroxytryptamine (Rapport, Green, and Page, 1948; Rapport, 1949).

Fig. 10 shows that 5-hydroxytryptamine exerts a depressor effect on the rat's blood pressure. This effect may be reduced by intravenous injection of hexamethonium, or dibenamine, or both, but it cannot be abolished.

It is unlikely that, if care is taken in the collection of the plasma, the small quantities of serotonin or histamine possibly present would be sufficient to interfere with the pressor assays. Landgrebe, Macaulay, and Waring (1946) have shown that relatively large doses of histamine are required to depress the blood pressure in the rat preparation. On the other hand, if present in sufficient amount, both serotonin and histamine

would tend to lead to an underestimate of the pressor activity.

Discussion

These results seem to demonstrate that the rat blood-pressure preparation, treated with dibenamine, is a simple, sensitive, and relatively specific method for the quantitative assay of angiotonin, and of the angiotonin-like activity of pathological plasma or plasma extracts. The pressor response is not interfered with by the possible presence of small amounts of pressor organic bases, nor by that of small amounts of vasopressin. Possible interference in the assay by the presence of small amounts of pressor organic bases is eliminated by the use of dibenamine, while possible interference by the presence of vasopressin can be avoided by inactivation of this substance by treatment of the sample with sodium thioglycollate.

Summary

1. A four-point procedure for the pressor assay of angiotonin, and the angiotonin-like activity of various samples of plasma and plasma extracts, has been developed using the rat blood-pressure preparation treated with dibenamine.

2. The pressor actions of the angiotonin standard and of the plasma samples were not due to adrenaline, noradrenaline, isoamylamine, tyramine, piperidine, or nicotine, since they survived the injection of dibenamine. The presssor actions were also not due to pituitary, since they survived treatment with 0.01 M-sodium thioglycollate.

3. The angiotonin solution contained pressor activity equivalent to about 100 mu. pituitary standard/ml., and oxytocic activity equivalent to about 7.5 mu. pituitary standard/ml.

4. The sensitivity of the rat blood-pressure preparation is about 20 times that of the cat preparation and about 80 times that of the dog preparation.

This work was carried out at the suggestion of Dr. G. C. Arneil of the Royal Hospital for Sick Children, Glasgow, whom I wish to thank for co-operating in the investigation of clinical aspects of this research which we are publishing separately. I am grateful to Dr. O. M. Helmer of the Eli Lilly Laboratory for Clinical Research, U.S.A., for a generous supply of "Solution Angiotonin" for this investigation. I am also grateful to Miss Margaret Harvie for her valuable assistance with the statistical work.

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1 : 6 - DI - 4′ - CHLOROPHENYLDIGUANIDOHEXANE ("HIBITANE"*). LABORATORY INVESTIGATION OF A NEW ANTIBACTERIAL AGENT OF HIGH POTENCY

BY

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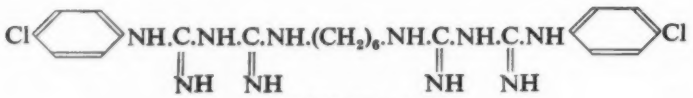
During an investigation of the biological properties of certain polydiguanides it was found that bisdiguanides of the general type:

aryl.NH.C(:NH).NH.C(:NH).NH.$(CH_2)_n$.
NH.C.(:NH).NH.C(:NH).NH.*aryl*‡

exhibited marked antibacterial activity *in vitro* against a wide range of micro-organisms, including *Pseudomonas pyocyanea*. Such bisdiguanides offered considerable scope for structural variation, and many substances of this kind were synthesized and tested (Table I) before it was ultimately decided that one compound, Serial No. 10,040, had the most outstanding bacteriostatic properties.



10,040 (Base)

This compound, in one form or another, has since been the subject of extensive laboratory, clinical, and veterinary study. The present communication is intended to record some of the background to the discovery of 10,040, and to indicate its useful potentialities through a brief account of the initial experimental observations made in these laboratories.

Two salts of the bisdiguanide 10,040, itself a colourless strongly basic substance, m.p. 134° C., were selected for study. One was the diacetate, m.p. 154° C., soluble to approximately 1.9% in distilled water at 20° C., and the second the dihydrochloride, m.p. 257° C., having a corresponding solubility of only 0.06%. The diacetate was clearly the more convenient for the preparation of stock solutions for laboratory work and for that reason has been used almost entirely throughout this research, although repeated tests *in vitro* have shown that the lower solubility of the dihydrochloride does not alter its antibacterial potency.

In addition to the determination of the antibacterial properties of 10,040, a number of other investigations, bearing on the potential applications of the agent, have been undertaken. These have included tests for compatibility with antibiotics such as penicillin; the effect of various possible vehicles known to support bacterial growth, such as milk and serum; the influence of *p*H, size of inoculum, presence of detergent substances; and the action of the drug on bacterial spores. The possible development of resistant organisms has also been studied. Finally, the behaviour in and towards the animal host has been followed by examining the influence of 10,040 on phagocytosis of bacteria by human leucocytes; by feeding experiments in rats extending over several generations; and by its local application to infected artificial wounds in mice.

METHODS

Measurement of Bacteriostatic Action.—Dilutions of the compound were made in Difco brain–heart infusion. Each tube was inoculated with one loopful of a 24 hr. culture of the test bacteria. The results were read after incubation for 24 hr. at 37° C.

Measurement of Bactericidal Action.—For this it was necessary to have available a substance which would neutralize, more or less completely, the action of the drug on the test bacteria in the medium used for subculture. It was ultimately found that egg-yolk (see Table III) contained such a compound, and this was applied as follows:

(*a*) *Preparation of Egg-yolk Solution.*—The yolks of six eggs were separated from the whites and

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‡ Patent protection pending.

emulsified with 600 ml. of Difco brain–heart infusion; 20 g. of kaolin was stirred in and the solution clarified by filtration through paper and then sterilized by Seitz-filtration.

(*b*) *Performance of Test.*—Standard inocula were prepared by growing the bacteria on the surface of 5 ml. of nutrient agar in a 50 ml. conical flask and suspending the growth in water with the aid of a few glass beads, as described by Davies (1951). Five ml. of this suspension of bacteria, diluted as required, was mixed with 5 ml. of the test solution. After the desired period of contact, 1 ml. of the mixture was added to 5 ml. of egg-yolk solution and 1 ml. samples of the inactivated solution plated out to obtain viable counts.

Action of 10,040 on Bacterial Spores.—Several strains of sporing bacilli were isolated freshly from garden soil. They all resisted the action of boiling water for 1 hr. Pure cultures of the bacilli were grown for 4 days on agar, by which time nearly all the cells had spored. Growth was washed off the agar with sterile distilled water and the suspension heated at 80–90° C. for 15 min. The heated suspension was then adjusted to contain 10,000,000 spores per ml. as determined by haemocytometer counts. These spores were suspended in 1% and 0.1% solutions of 10,040 to give final concentrations of 1,000,000 spores per ml. After varying intervals of time samples were inactivated with egg-yolk and tested for the presence of viable spores.

Treatment of Artificial Wounds in Mice.—The technique of Gordon *et al.* (1947) was used. An ellipse of skin was cut from the back of a mouse anaesthetized with ether, and 2 drops of a 24 hr. culture of Group A streptococci (Kruger) dropped on to the exposed fascia, which was then lightly scarified. Fifteen minutes later 2 drops of the test solution were dropped into the wound and the skin closed with a single Michel clip.

Therapeutic Activity in Mice.—Groups of 10 mice were infected intraperitoneally with 0.2 ml. of a 10^{-4} dilution of a blood broth culture of *Streptococcus pyogenes*. The mice were treated with 0.2 mg. per 20 g. of 10,040 diacetate intraperitoneally, treatment being given just before, and at various periods after, infection.

Results

Bacteriostatic Action.—Table I provides a selection of the several hundred substances prepared during the course of this work, and illustrates the connexion between chemical structure and bacteriostatic effect. Activity in each compound is related to 10,040 as standard. The data for the first three compounds (12,483, 10,040, and 11,383) showed the influence of the length of the polymethylene chain and, with other results not recorded here, illustrated the superior effect provided by hexamethylene. Other compounds were made using different kinds of conjunctive groupings—for example, aromatic and mixed aromatic-aliphatic systems such as those in 11,384 and 11,385—but in general these were much less active. It was also found that the solubility in water, of bisdiguanides varied in this manner, was often greatly diminished. Many variations were made in the type of group or groups substituted in the terminal benzene rings. The relative activities of a few of these preparations are shown in Table I, selecting for convenience of comparison those based on the hexamethylene conjunctive system. Omission of the chlorine atoms (10,387), or the introduction of a second halogen into each benzene ring (11,386), reduced activity, while the depressant effect of the acidic hydroxyl and carboxyl groups, in 11,108 and 10,691 respectively, was

TABLE I

COMPARATIVE BACTERIOSTATIC ACTIVITY OF A NUMBER OF AGENTS RELATED TO 10,040

Compounds of type: R.NH.C.NH.C.NH(X)NH.C.NH.C.NH.R (NH, NH, NH, NH)

Code No.	Terminal Group R	Central Unit X	Comparative Bacteriostatic Effect		
			Bact. coli	*Staph. aureus*	*Ps. pyocyanea*
12,483	4-Chlorophenyl	Trimethylene	0·3	1	0·3
10,040	"	Hexamethylene	1	1	1
11,383	"	Decamethylene	0·3	0·3–1	<0·01
11,385	"	(4:4′)-Diphenylmethane	1	0·3	0·3
11,384	"	(1:4)-Phenylene	0·1	0·1	0·03
10,387	Phenyl	Hexamethylene	0·3–1	0·3–1	0·1
11,386	3:4-Dichlorophenyl	"	0·3	0·3	1
11,108	4-Hydroxyphenyl	"	0·03	0·01	<0·01
10,689	4-Methoxyphenyl	"	0·3	0·1	0·01
10,691	4-Carboxyphenyl	"	<0·01	<0·01	<0·01
9,381	RNH.=Et_2N	"	0·1	0·3	<0·01
14,575	RNH.=4-ClC_6H_4NMe-	"	0·3	1	0·1
10,030	RNH.C.(NH)=hydrogen	"	<0·01	<0·01	<0·01
11,717	RNH.C.(NH)=4-chlorophenyl	"	0·03	0·03	0·03

especially marked. The need for the terminal aromatic nucleus was illustrated by the antibacterial results obtained with the bisdiguanide 9,381, while the comparative uselessness of the guanidine system—in contrast to that of the diguanide—was shown with compounds 10,030 and 11,717.

The bacteriostatic activity of 10,040 against a wider range of organisms is shown in Table II. Gram-negative bacteria were sensitive to its action, but, on the whole, they were rather less sensitive than Gram-positive species.

TABLE II

BACTERIOSTATIC ACTION OF 10,040 ON VARIOUS BACTERIA IN HEART-BRAIN INFUSION

Organism	Minimal Concn. Inhibiting Growth at 37° C. in 24 hr.
Bacillus subtilis (1)	1 : 1,000,000
Streptococcus lactis (1)	1 : 2,000,000
Streptococcus pyogenes (4)	1 : 500,000–1 : 1,000,000
Corynebacterium diphtheriae (1)	1 : 500,000
Streptococcus pneumoniae (1)	1 : 500,000–1 : 1,000,000
Staphylococcus aureus (20)	1 : 500,000–1 : 2,000,000
Proteus vulgaris (1)	1 : 200,000
Salmonella pullorum (1)	1 : 300,000
Vibrio cholerae (1)	1 : 200,000
Streptococcus faecalis (1)	1 : 200,000
Salmonella dublin (1)	1 : 100,000
Salmonella typhi-murium (1)	1 : 100,000
Aerobacter aerogenes (1)	1 : 100,000
Bacterium coli (1)	1 : 100,000
Pseudomonas pyocyanea (10)	1 : 50,000–1 : 100,000

The figures in brackets indicate the number of strains tested.

In most bacteriostatic tests the transition from no growth to full growth was sharp. Occasionally, when the test organisms were staphylococci, a number of the tubes, which at first sight appeared clear, were found to contain a tiny white speck of growth at the bottom. When the supernatant broth was removed by centrifugation, and replaced by fresh broth not containing 10,040, the bacteria divided further until they produced full normal turbidity. These trace growths appeared at random throughout the series. Their appearance bore no relation to the concentration of 10,040 over a fairly wide range. Further experiments on these survivors are now in progress.

Neutralization.—The powerful inhibition of the bacteriostatic activity of 10,040 by broth containing egg-yolk, and the ultimate use of this phenomenon to distinguish between bacteriostatic and bactericidal effects, has already been commented upon. The earliest attempts to achieve this end concerned the use of simple anions such as sulphate, which might, for example, have caused deactivation of the agent by precipitation of the very sparingly soluble disulphate ; but no demonstrable difference in growth was observed. Many other substances examined for the same purpose (Table III) also permitted the retention of a considerable degree of bacteriostatic activity. These included serum, nucleic acid, milk, suramin (chosen as a polyanionic agent), and Lubrol W (which neutralizes quaternary ammonium compounds). It will be noted that lecithin was without action.

TABLE III

THE EFFECT OF VARIOUS SUBSTANCES ON THE BACTERIOSTATIC ACTIVITY OF 10,040 ON *STAPH. AUREUS B.E.*

Substance Added to Heart-Brain Infusion	Minimal Inhibitory Concn. of 10,040
None	1 : 500,000
1% yeast nucleic acid	1 : 500,000
1% suramin	1 : 200,000
0·1% thioglycollic acid	1 : 500,000
10% full cream evaporated milk	1 : 100,000
10% rabbit serum	1 : 200,000
1% Lubrol W	1 : 200,000
1% Tween 80	1 : 500,000
0·1% egg lecithin	1 : 500,000
Egg-yolk (1 yolk per 100 ml.)	1 : 4,000

Bactericidal Action.—The bactericidal action of 10,040 was unusual. A very high proportion of the inoculum was killed within a short time even at high dilutions. Much higher concentrations were required to produce a 100% kill.

TABLE IV

BACTERICIDAL ACTION: EFFECT OF VARYING THE CONCENTRATION OF 10,040 ON THE NUMBER OF SURVIVING BACTERIA. *STAPH. AUREUS B.E.*: *p*H 7.0, 20° C.

Concn. of 10,040	No. of Bacteria per ml. Surviving		
	0 min.	5 min.	10 min.
1 : 20,000	2×10^8	200	70
1 : 30,000	2×10^8	4,000	200
1 : 40,000	2×10^8	600	5
1 : 50,000	2×10^8	11,000	0
1 : 60,000	2×10^8	2,500	40
1 : 70,000	2×10^8	40,000	150
1 : 80,000	2×10^8	7,000	120
1 : 90,000	2×10^8	5,000	800
1 : 100,000	2×10^8	23,000	2,000
1 : 200,000	2×10^8	150,000	600

Table IV illustrates this phenomenon. A concentration of 1 : 200,000 of 10,040 killed more than 99.9% of the bacteria present in 5 min., but 1 : 20,000 did not kill completely in 10 min. Reduction in the size of inoculum, with any given period of contact between 10,040 and the bacteria still resulted in the appearance of substantially the same number of survivors (Table V). The first hypothesis advanced to account for the survivors was that they represented a few cells which were naturally resistant to the action of 10,040. When, however, the survivors were re-grown and re-tested, the same phenomenon appeared. The bactericidal action was influenced by the *p*H of

TABLE V

BACTERICIDAL ACTION: EFFECT OF INOCULUM-SIZE ON THE NUMBER OF SURVIVING BACTERIA. *STAPH. AUREUS B.E.*: *p*H 7.0, 20° C.; 1: 20,000 OF 10,040

No. of Bacteria per ml. Added	No. of Bacteria per ml. Surviving After		
	5 min.	15 min.	60 min.
6×10^8	1,300	40	4
6×10^6	1,200	150	15
6×10^3	70	14	8

TABLE VI

BACTERICIDAL ACTION: INFLUENCE OF *p*H ON THE NUMBER OF SURVIVING BACTERIA. *STAPH. AUREUS B.E.*: 20° C.; 1: 20,000 OF 10,040

*p*H of Solution	No. of Bacteria per ml. Surviving After		
	0 min.	5 min.	15 min.
5·25	4,000,000	>1,000,000	>1,000,000
6·22	4,000,000	2,300,000	50,000
7·08	4,000,000	90,000	5,000
7·97	4,000,000	17,000	900

NOTE: The 10,040 was dissolved in M/15 phosphate buffer; portions of the solution were adjusted to the desired *p*H using a glass electrode.

the solution ; the activity increased with increasing alkalinity (Table VI).

Compatibility with Antibiotics.—10,040 is compatible with penicillin, streptomycin, chloramphenicol, oxytetracycline, and aureomycin. With penicillin, for example, bacteriostatic assays were made against *Staphylococcus aureus A.B.* and *A.C.*, using six serial dilutions of penicillin ranging from 0 to 0.25 u./ml., divided into four groups each containing concentrations of 10,040 of 0, 0.1, 0.5, and 1.0 μg./ml., respectively. In no case was there any indication that the action of the one agent was influenced by the presence of the other.

The Toxicity of 10,040.—The acute toxicity of 10,040 given by various routes to mice is shown in Table VII. The mice received one dose of the compound and were observed for a period of 10 days.

The compound appeared to have a very low toxicity when given orally to rats over long periods.

TABLE VII

THE ACUTE TOXICITY OF 10,040 IN MICE

(Albino mice weighing 16–22 g. given one dose of the compound and observed for ten days. The numbers of mice used are given in parentheses.)

Salt of 10,040	Route of Administration	Weighted Mean LD50 (mg./kg.) ± S.E.
Dihydrochloride	Subcutaneously in 0·5 ml.	>5,000 (40)
Diacetate ..	,, ,, 0·5 ,,	325 ± 201 (20)
,, ..	Intraperitoneally in 0·1 ,,	38 ± 3·8 (95)
,, ..	Intravenously in 0·1 ml.	25 ± 2·0 (90)
,, ..	Orally in 0·5 ml.	2,000 ± 580 (100)

Two groups of rats, each of 12 females and 2 males, were selected. The control group was given water to drink and the other group received a 1 in 2,000 solution of 10,040 diacetate as its only source of water. Litters were obtained from all the females. Two litters were produced from these animals of the second generation, the dosing with 10,040 being maintained throughout. The original parents had been drinking 10,040 for one year when the experiment ended.

The rats were weighed weekly throughout the experiment. Every three months a total and differential blood-cell count was made and the blood haemoglobin was estimated. At the end of the experiment the rats were killed and the tissues examined histologically. In every respect the treated animals were similar to the controls.

Effect on Phagocytosis.—A 1 : 10,000 solution of 10,040 in normal saline did not affect the phagocytosis of staphylococci by human leucocytes when tested by the method of Davies (1951).

Action on Bacterial Spores and on Mycobacterium tuberculosis.—No spores were recovered from a 1% solution of 10,040 after 2 hr. at room temperature, but a 0.1% solution had caused no reduction in numbers after 24 hr. at room temperature.

10,040 inhibited the growth of *M. tuberculosis* at 1 in 1,000,000 in the medium of Dubos and Middlebrook (1947). It was inactive at 1 in 1,000 on Löwenstein's medium (which contains egg-yolk).

Failure of Bacteria to Develop Resistance to 10,040.—Many attempts have been made to demonstrate bacterial resistance to 10,040, but without success. Serial transfers were made in a variety of media with several strains, both of *Staph. aureus* and *Ps. pyocyanea.* No increase in resistance was noted.

The Action of 10,040 in the Treatment of Artificial Wounds in Mice.—Table VIII shows that 10,040 exerted a marked action on artificial wounds in mice infected with pathogenic streptococci. Fourteen out of 15 mice treated with 1%

TABLE VIII

THE EFFECT OF 10,040 AND PROFLAVINE APPLIED TO ARTIFICIAL WOUNDS INFECTED WITH STREPTOCOCCI

Application	No. of Mice	Mean Survival Time in Hr. (max. 120 hr.)	No. of Survivors at 5 Days
1% 10,040 ..	15	117	14
1% proflavine ..	15	60	3
Controls	15	35	0

10,040 survived for five days. All 15 control mice died with a mean survival time of 35 hr. Proflavine (1%) exerted very little effect under the same conditions.

Therapeutic Activity in Mice.—Table IX shows that 10,040 exerted a slight but definite therapeutic effect against a streptococcal infection in mice when both drug and infection were given intraperitoneally. Treatment given 1, 6, 24, and 30 hr. after infection was no more effective than a single treatment given 1 hr. after infection. Thus 10,040 does not appear to influence those organisms which survive contact with it in the peritoneal cavity and subsequently invade the body. No therapeutic activity was demonstrable when 10,040 was given by the oral or subcutaneous routes to mice infected intraperitoneally.

TABLE IX

THE EFFECT OF 10,040 ON THE SURVIVAL TIME OF MICE INFECTED WITH STREPTOCOCCI

Interval between Dosing and Infection	Expt. No.	No. of Deaths on Day					Total Deaths	Mean Survival Time* (Days)
		1	2	3	4	5		
Dosed 1 hr. before	256	1	9				15/20	3·5
	258		4		1			
„ 1 min. „ ..	256		6	4			20/20	2·3
	258		9	1				
„ 1 „ after ..	256		7	1	1	1	16/20	3·5
	258		6					
„ 1 hr. „ ..	256		8	1		1	19/20	2·6
	258		7	2				
„ 2 „ „ ..	256	1	7	2			19/20	2·3
	258	1	8					
„ 4 „ „ ..	256	5	5				20/20	1·8
	258		9		1			
„ 1, 6, 24, and 30 hr. after	256		8	2			19/20	2·7
	258		5	3	1			
Untreated controls	256	8	2				20/20	1·1
	258	10						

* Mice dying on Day 1 allotted a survival time of 1 day; on Day 2 of 2 days, and so on. No mice died on the 6th or the 7th day; all those alive at 7 days were allotted a survival time of 8 days.

SUMMARY

1. A bisdiguanide, 10,040, " Hibitane," 1:6-di-4'-chlorophenyldiguanidohexane, was selected from an extensive series of related compounds as the one possessing the highest degree of antibacterial activity.

2. Its antibacterial action is exerted against a wide range of vegetative bacteria both Gram-positive and Gram-negative.

3. The compound is feebly active against bacterial spores.

4. Activity is maintained in the presence of body-fluids but is antagonized by a substance contained in egg-yolk.

5. Attempts to demonstrate development of drug-resistance were unsuccessful.

6. 10,040 has a very low oral toxicity for laboratory animals and does not interfere with phagocytosis by human leucocytes.

7. It has no true systemic antibacterial activity in mice but is highly active in artificial wounds infected with haemolytic streptococci.

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COMPARISON OF HISTAMINE RELEASE BY COMPOUND 48/80 AND OCTYLAMINE IN PERFUSED TISSUES

BY

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It has recently been shown that a series of simple mono-amines are histamine releasers. The histamine release was studied in incubated tissue particles of guinea-pig lung, and octylamine was found to be several times more active than 48/80. When, however, the activity of these compounds in producing the triple response on the human skin *in vivo* was measured, the result was different. The mono-amines were active in about the same concentrations as on the tissue particles, but 48/80 was nearly a thousand times more active than octylamine. This discrepancy raised the question as to whether the activity of a histamine releaser varies when examined by different methods (Mongar and Schild, 1953). In the present experiments, the activity of 48/80 and octylamine was therefore compared by another method widely used in the past for the study of histamine releasers—namely, the perfusion of isolated tissues.

METHODS

The tissues perfused were the skin of the hind legs and gastrocnemius muscle of the cat, the hindquarters of rats and guinea-pigs, and lungs of all three species. The cats were anaesthetized with chloralose intravenously, and the rats and guinea-pigs with pentobarbitone sodium intraperitoneally. The skin flaps of the hind legs and of gastrocnemius muscles of the cat were perfused by the method described by Feldberg and Paton (1951) and Feldberg and Schachter (1952). For the perfusion of the hindquarters a cannula was inserted into the lower abdominal aorta and the venous outflow collected from a cannula in the vena cava. A mass ligature was firmly tied round the body proximal to the cannulation sites and the upper half of the animal cut off. For perfusion of the lungs the pulmonary artery was cannulated. In the experiments on rats and guinea-pigs the lungs were removed from the body and placed in a jacketed funnel at 37° C., the trachea was cannulated, the lungs were inflated with air and the venous effluent allowed to flow out into the funnel through an incision in the left auricle. In the experiments on cats the lungs were kept *in situ* and rhythmically inflated by an artificial respiration pump ; both the pulmonary artery and vein were cannulated and the venous outflow collected through the venous cannula. The perfusion fluid was warm, aerated Locke or Tyrode solution.

The histamine in the venous samples was assayed on the atropinized guinea-pig's ileum. All values refer to the base.

Octylamine was obtained as the free base from Light & Co. The hydrochloride was prepared by suspending the oily liquid in about 10 times the volume of distilled water and titrating with conc. HCl till neutral. This solution of hydrochloride is stable. Compound 48/80 was obtained through the kindness of the late Dr. C. H. Kellaway of the Wellcome Research Institution. Both octylamine and 48/80 were injected, if not otherwise stated, in a volume of 0.5 ml.

RESULTS

Perfusion of Cat Tissues

Skin Flaps.—Weight for weight, the histamine-releasing activity of 48/80 in the perfused cat's skin preparation is about 200 times as strong as that of octylamine.

In one experiment, 50 μg. octylamine released 18.3 μg., and a subsequent injection of 0.5 μg. 48/80, 22 μg. of histamine. Since a second injection produces on the same skin flap a reduced release of histamine, 48/80 is certainly more than 100 times as active as octylamine. In two experiments the substances were compared by injecting one into a skin flap from the right, and the other into that from the left hind leg of the same cat. In the one experiment, 5 μg. octylamine released 0.58 μg., and 0.5 μg. 48/80 released 30.6 μg., histamine ; in the other experiment, 50 μg. octylamine released 10.1 μg. from the right, and 0.2 μg. 48/80 released 4.7 μg. histamine from the left, skin flap. This experiment is shown in Fig. 1, which also illustrates that successive injections of histamine liberator produce diminishing effects. From the three experiments it can be concluded that 48/80

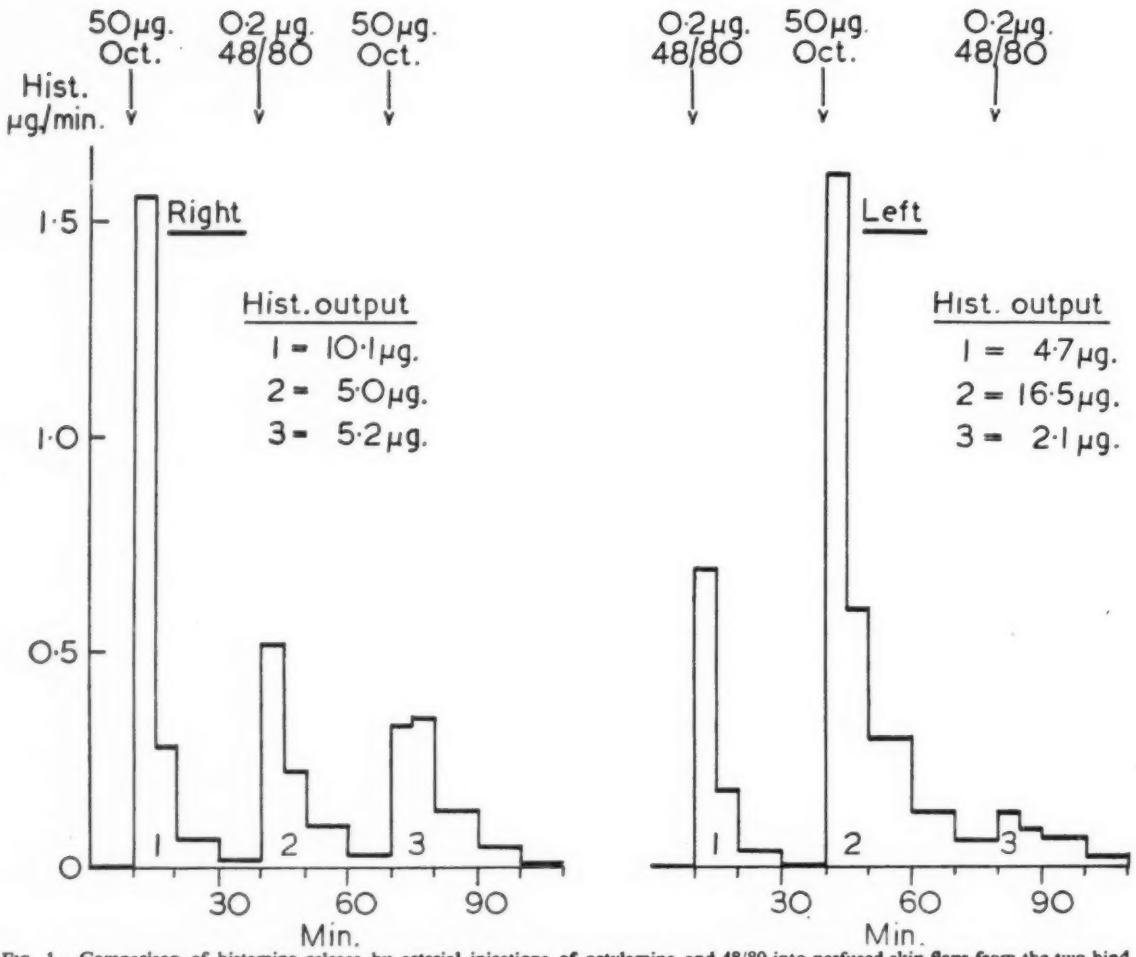

FIG. 1.—Comparison of histamine release by arterial injections of octylamine and 48/80 into perfused skin flaps from the two hind limbs of a cat. 50 μg. octylamine releases more histamine than 0.2 μg. 48/80.

is considerably more than 100 and rather less than 250 times as active as octylamine.

In order to find out if a different result would be obtained when octylamine and 48/80 were left in contact with the skin for a longer period than that attained on single arterial injection, the substances were given by infusion. In one experiment, one skin flap was infused with 100 μg./ml. octylamine, the other with 1 μg./ml. 48/80. The amounts of histamine released during 100 min. infusion were 137 and 210 μg. respectively. In another experiment the concentrations used for infusion were 20 μg./ml. octylamine and 0.1 μg./ml. 48/80. This experiment is illustrated in Fig. 2. The histamine output during 100 min. infusion was 39 and 51 μg. respectively, showing that, weight for weight, 48/80 is again about 200 times as active as octylamine. In Fig. 3 the amounts of histamine collected in the successive samples are plotted, for both infusion experiments, as percentages of the total release. When the results are expressed in this way, it becomes clear that the initial rate of output is greater with 48/80 than with octylamine. For instance, after 25 min. infusion, the percentages of the total histamine collected in the two experiments were 52 and 62% with 48/80 and 38 and 34% with octylamine.

TABLE I

OUTPUT OF HISTAMINE FROM PERFUSED GASTROCNEMIUS MUSCLES OF THE SAME CAT BY ALTERNATE INJECTIONS OF 48/80 AND OCTYLAMINE

Order of Injection	Right Gastrocnemius		Left Gastrocnemius	
	Injection	Output of Histamine (μg.)	Injection	Output of Histamine (μg.)
1st	5 μg. 48/80	3·7	1,000 μg. octylamine	3·6
2nd	500 μg. octylamine	0·2	5 μg. 48/80	2·1
3rd	5 μg. 48/80	0·3	1,000 μg. octylamine	0·4

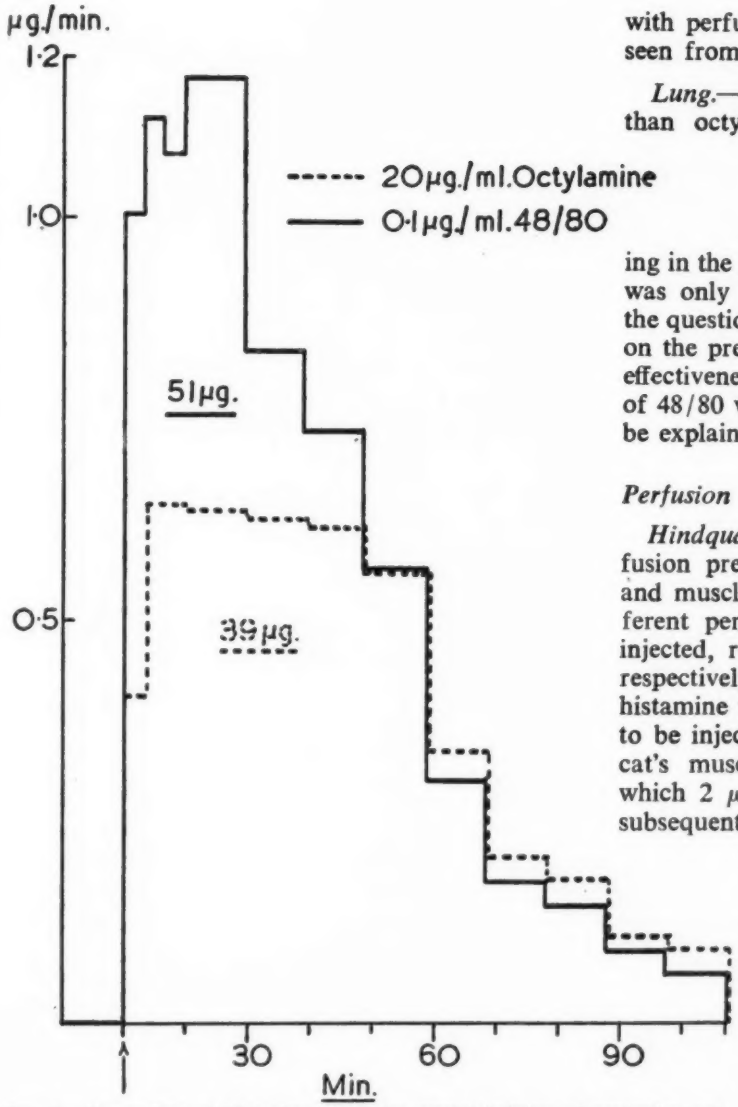

FIG. 2.—Comparison of histamine release by arterial infusion of octylamine and 48/80 into perfused skin flaps from the two hind limbs of a cat. Infusion of 20 µg./ml. octylamine releases less histamine than does 0.1 µg./ml. 48/80.

Gastrocnemius Muscle.—The relative activity of 48/80 to octylamine in releasing histamine from the perfused gastrocnemius muscle is the same as with perfused skin flaps—i.e., about 200. This is seen from the experiment summarized in Table I.

Lung.—Compound 48/80 was more effective than octylamine in releasing histamine in this tissue, but was not as active as on skin and muscle preparations. This is illustrated by the results of the two experiments given in Table II. The finding in the second experiment that 2 mg. octylamine was only effective when given after 48/80 raises the question of how far this release was dependent on the previous action of 48/80. Further, the ineffectiveness in both experiments of a second dose of 48/80 which had been previously active cannot be explained by depletion of the tissue histamine.

Perfusion of Rat Tissues

Hindquarters (*Muscle and Skin*).—In this perfusion preparation, as in the perfused cat's skin and muscle, 48/80 is highly active. In three different perfusions, 1, 2, and 10 µg. 48/80 were injected, releasing 2.8, 4.2, and 28 µg. histamine respectively. In order to produce a release of histamine with octylamine, much larger doses had to be injected, as in the perfusion experiments of cat's muscle and skin. In the experiment in which 2 µg. 48/80 released 4.2 µg. histamine, a subsequent injection of 200 µg. octylamine released 1.9 µg. histamine; in the experiment in which 1 µg. 48/80 released 2.8 µg. histamine, a previous injection of 500 µg. octylamine had only released 0.3 µg. histamine. It was not possible to obtain a large histamine release by increasing the dose of octylamine to 5 mg.; this dose, which caused intense reduction in venous outflow and oedema, produced an output of only 2 µg. histamine. From these results it appears that the difference of activity of octylamine and 48/80 in this preparation is greater than on perfused cat

TABLE II

OUTPUT OF HISTAMINE FROM PERFUSED CAT'S LUNGS AFTER ALTERNATE INJECTIONS OF OCTYLAMINE AND 48/80

1st Experiment		2nd Experiment	
Injection	Output of Histamine (µg.)	Injection	Output of Histamine (µg.)
1 mg. octylamine	Nil	2 mg. octylamine	Nil
1 „ 48/80	21·3	0·1 „ 48/80	11·5
5 „ octylamine	2·8	2 „ octylamine	2.5
1 „ 48/80	Nil	0·1 „ 48/80	Nil

TABLE III

OUTPUT OF HISTAMINE FROM PERFUSED RAT LUNGS AFTER ALTERNATE INJECTIONS OF OCTYLAMINE AND 48/80

1st Experiment		2nd Experiment	
Injection	Output of Histamine (µg.)	Injection	Output of Histamine (µg.)
1 mg. octylamine	5·9	1 mg. octylamine	1·2
0·5 „ 48/80	3·8	0·5 „ 48·80	2·6
1 „ octylamine	1·4	1 „ octylamine	0·5
0·5 „ 48/80	1·8	0·25 „ 48/80	0·3
1 „ octylamine	0·9	1 „ octylamine	0·4

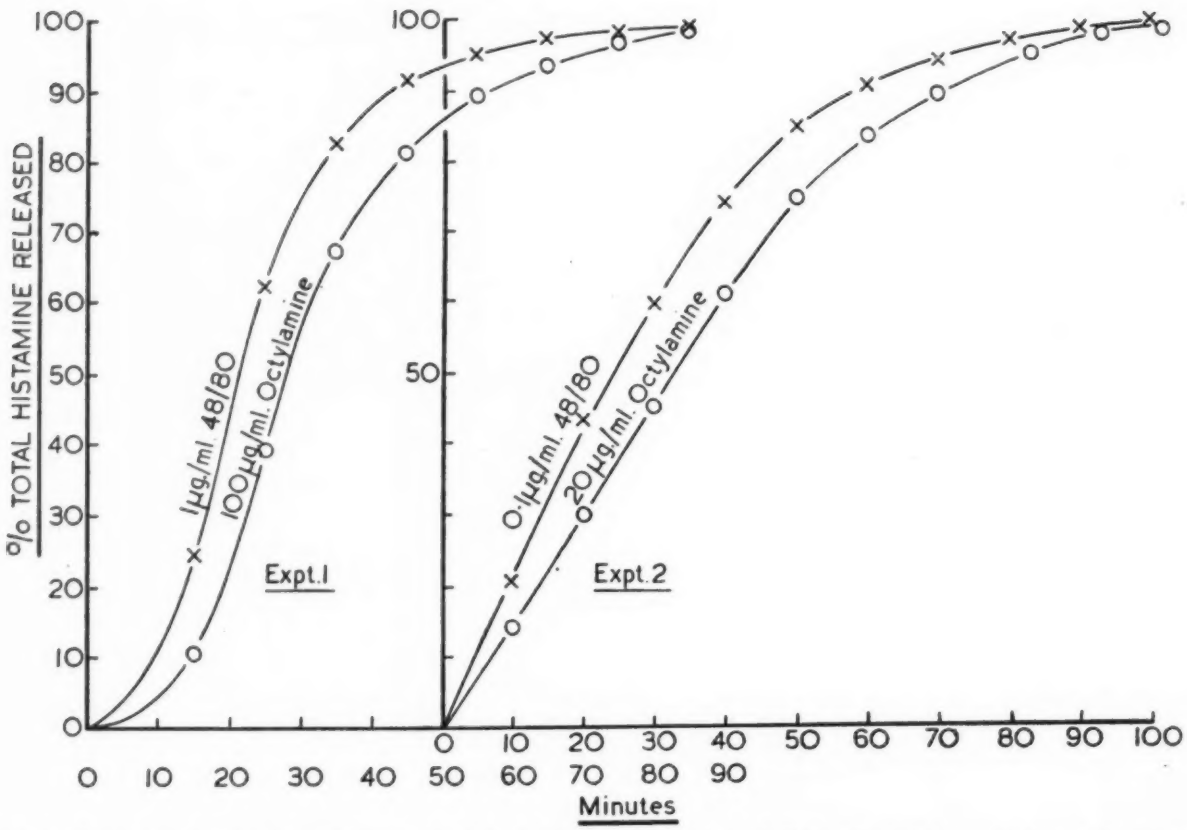

FIG. 3.—Comparison of histamine release curves obtained by infusion of octylamine and 48/80. The initial rate of release is faster with 48/80 than with octylamine. Each comparison was made on perfused skin flaps from the two hind limbs of a cat. Expt. 2, same as that of Fig. 2.

tissue, and resembles more that observed on the human skin by Mongar and Schild (1953).

It is worth mentioning that the development of oedema was much more pronounced after large doses of octylamine than after doses of 48/80 which gave larger releases of histamine.

Lung.—On this perfused tissue the difference in the potency of 48/80 and octylamine was not pronounced. Compound 48/80 was about two to four times as active as octylamine, as seen from the results in Table III.

Perfusion of Guinea-pig Tissues

Hindquarters (Muscle and Skin).—On this preparation 48/80 is not as active as on the rat's perfused hindquarters. The threshold amount of 48/80 was about 20 µg., and even 50 µg. usually released less than 1 µg. histamine. For instance, in one experiment three successive doses of 50 µg. 48/80, injected at 10-min. intervals, released 0.28, 0.43, and 0.32 µg. histamine, and a subsequent fourth injection of 500 µg. 48/80 released 3.4 µg.

When compared with octylamine, it was found that 48/80 was about 60 times more active in releasing histamine. This is illustrated by the results of two perfusion experiments in which the effect of 2 mg. octylamine was compared in one with 50 µg., and in the other with 25 µg., 48/80. The output of histamine in µg. was 1.1, 0.3, 1.3 in the first experiment with the higher dose of 48/80, and 0.2, 0.7, 0.1 in the second experiment. The underlined values refer to the histamine released by the 2 mg. octylamine.

Lung.—With doses of 0.5 to 1 mg. of either 48/80 or octylamine, usually only a fraction of a µg. (between 0.02 and 0.8 µg.) was released from this perfused tissue. Only in one out of eight experiments was the release of histamine by both octylamine and 48/80, when injected in doses of 1 mg., greater than 1 µg. The ability to release histamine appeared to be of the same order with the two substances. Thus in eight experiments, octylamine gave a greater release in three, and

48/80 in two; in the remaining three there was no appreciable difference when the two releasers were given alternately to the same preparation.

Discussion

A comparison of the effectiveness of 48/80 and octylamine in releasing histamine gives a different result when it is made on perfused skin and muscle preparations from that when it is made on perfused lungs. On skin and muscle 48/80 is many times more active than octylamine—at least 1,000 times in the rat, about 200 in the cat, and about 60 in the guinea-pig—and a release can be obtained with a few μg. and, on some preparations, such as the cat's skin, with a fraction of a μg. of 48/80. On the perfused lungs, however, 48/80 as well as octylamine had usually to be injected in doses of the order of 1 mg. to release measurable amounts of histamine, though cat lung appeared to release more readily to 48/80 than did rat and guinea-pig lung. The activities relative to octylamine are about 20, 3, and 1 respectively. Thus, a comparison of the activity of two releasers depends to a great extent on the species and tissue used for perfusion.

Apart from the species and tissue differences seen when comparing the activity of histamine releasers, the results will also be greatly influenced by the method used for their assay. If, instead of perfusion experiments, the histamine releasers are allowed to act in incubation experiments on tissue particles, the relative activities are different, For instance, Mongar and Schild (1953) found that with this method octylamine was more active than 48/80, when allowed to act on guinea-pig's lung particles. It is not clear why different methods give such different results, but the unique property of 48/80 in releasing histamine, when tested in very low concentrations, either in perfusion experiments or on the intact human skin, is no longer shown when tested in incubation experiments on skin particles (Mongar and Schild, unpublished experiments).

It is evident from our results that when the activities of two histamine releasers are compared, different ratios can be obtained according to the species, tissues, and methods used. This has to be kept in mind in the assay of histamine releasers. The problem is even more complicated, since the relative ability to release histamine in different species and tissues, and with different methods, may be different for each compound, so that the results obtained using one compound as a standard may have no relation to those obtained when a different reference compound is used.

Summary

1. The histamine-releasing activities of compound 48/80 and octylamine were compared on perfused skin, muscle, and lung of the cat, rat, and guinea-pig.

2. On skin flaps and gastrocnemius muscle of the cat, 48/80 was about 200 times more active than octylamine. On the perfused hindquarters preparation of the rat 48/80 was over 1,000 times, and on that of the guinea-pig about 60 times, more active than octylamine.

3. On perfused lungs, 48/80 was 1 to 20 times more active than octylamine, depending on the species.

4. The importance of tissue, species, and method specificity in assaying histamine releasers is discussed in the light of these results.

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THE HYPERSENSITIVITY OF THE DENERVATED NICTITATING MEMBRANE TO VARIOUS SUBSTANCES

BY

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The hypersensitivity to adrenaline which develops in the nictitating membrane after removal of the superior cervical ganglion has long been known. In 1932 Rosenblueth showed that the change in sensitivity was not restricted to adrenaline, but also occurred when ACh, pilocarpine, eserine, or histamine were used to stimulate the membrane. In 1934 Bacq and Rosenblueth added to these calcium chloride and potassium chloride, which they said stimulated the denervated membrane after the adrenal glands were excluded from the circulation. Since the innervation of the nictitating membrane is sympathetic it seemed clear that the phenomenon of hypersensitivity could have no relation to the humoral transmitter of sympathetic impulses.

In the following year, however, Bacq and Fredericq (1935) published evidence that there were some cholinergic fibres present; they found that supramaximal stimulation of the cervical sympathetic chain caused a greater contraction of the nictitating membrane when eserine was instilled into the eye, and that when atropine was given later by intravenous injection the contraction following stimulation of the chain was diminished below the initial size. These observations made it possible that the hypersensitivity of the denervated membrane to ACh might be related to this cholinergic innervation. In 1936 Ross showed that in cats under allobarbitone (" Dial "), in which the adrenal glands were excluded from the circulation, the contractions of the denervated nictitating membrane caused by adrenaline, ACh, $CaCl_2$, or KCl were reduced by 933F and by yohimbine, which are ordinarily regarded as specific antagonists for adrenaline. It may be noted that the action of ACh was diminished less than that of the other three substances.

Gaddum and Goodwin (1947) in the course of other work investigated the behaviour of histamine in cats under allobarbitone to which cocaine was given and in which the adrenal glands were excluded. They found that to histamine " the membrane was usually about as sensitive as it was to adrenaline." Denervation " sensitized the membrane to small doses, but when the dose was increased the difference between the two effects disappeared and even became reversed for a very large dose."

Most of the foregoing observers have had in mind that the substances ACh, histamine, KCl, and pilocarpine liberate the amines of the adrenal medulla, and have excluded the adrenal glands. Recent work by Bein and Meier (1952) has suggested that histamine may release substances with an adrenaline-like action from other places. They removed the adrenal glands in the spinal cat and waited until an injection of histamine no longer produced a secondary rise of blood pressure after the primary fall. They then injected L-cysteine and observed a restoration of the secondary rise 45–60 min. later, the rise being much greater than that seen before adrenalectomy. They found that this secondary rise was absent if the renal vessels were clamped on both sides. Their observations did not prove that the secondary rise was due to the release of a pressor substance, but this seems the most likely explanation. If that is so, the action of ACh, histamine, pilocarpine, eserine, KCl, and $CaCl_2$ on the nictitating membranes may perhaps be indirect and due to the release of an adrenaline-like substance. Such an explanation would fit in well with the evidence that their action is augmented by cocaine and diminished by 933F and yohimbine.

A further investigation became necessary after the work of Burn and Robinson (1952, 1953), who found that the removal of the superior cervical ganglion in the cat led to a fall in amine oxidase in the nictitating membrane, and suggested that this fall was responsible for the increased action of adrenaline and noradrenaline. They supported

this view by evidence of a correlation between the extent of the fall in amine oxidase and the increase of hypersensitivity to noradrenaline which was obtained in a series of 25 cats. Thus they believed that the hypersensitivity was explained by the fall in the enzyme. But the statement of Cannon and Rosenblueth (1949) that " the supersensitivity of the denervated nictitating membrane to acetylcholine, pilocarpine, eserine, and histamine, as well as that to calcium and potassium salts cannot reasonably be attributed to a decrease of amine oxidase or of phenolases " remained unanswered.

The position of ACh was clarified by the evidence of Burn and Philpot (1953), who recorded the presence of both true and pseudo-cholinesterase in the nictitating membrane, and found that after removal of the superior cervical ganglion there was a fall in the amount of true cholinesterase. This fall would explain the hypersensitivity to ACh.

Accordingly, a study has been made of the action of histamine and other substances on the normal and denervated membranes of the cat both with the ordinary circulation and when the head was perfused with Locke's solution containing dextran. The results in the perfused preparation enabled us to see how far the action of the different substances was exerted directly on the membranes. The observations of Bacq and Fredericq (1935) have also been repeated.

Methods

Observations were made on cats under chloralose anaesthesia, under allobarbitone (" Dial ") anaesthesia and in spinal preparations. In most experiments the cats were prepared by excision of the right superior cervical ganglion 4–10 days previously. The contractions of the nictitating membrane were recorded by attaching them to isotonic levers fitted with frontal writing points ; the contractions were magnified 7.5 times.

The perfusion experiments were carried out by dissecting the two external jugular veins and the two carotid arteries, and passing two ligatures around each of these vessels. The next steps were taken as rapidly as possible. A clip was put on one jugular vein, and a cannula was inserted below the clip so as to lead away from the head. The other vein was treated similarly. Artery cannulae pointing to the head were then placed in the two carotids. A Dale–Schuster pump was used to pump the perfusion fluid to a Y-piece, the ends of which were connected to the two carotid cannulae by polythene tubing. Two polythene tubes were joined to the vein cannulae and led the effluent to a collecting jar. A 4-way cannula was placed on the path from the pump to the Y-piece, immediately before the Y-piece, so that a thermometer could be inserted, and the pressure recorded. The time from the interruption of the natural circulation to the beginning of the perfusion was 10–15 min. The perfusion fluid, which was well oxygenated beforehand, was a mixture of 1 part of a 6% dextran solution and 3 parts of Locke's solution. The temperature was 37°. Care was taken that the rate of outflow from the tubes leading from the veins was the same on the two sides ; this depended on the position of the cannulae. Injections were made into the perfusion fluid just before it reached the Y-piece, so that the injected material was distributed to the two sides evenly. Observations were almost always completed in 10–20 min. from the start of the perfusion.

When experiments were carried out in cats in which the cervical sympathetic chain was stimulated, the chain was laid on the two prongs of shielded electrodes and covered with liquid paraffin. In two experiments a unipolar electrode was gently hooked round the postganglionic fibres. Stimulation was provided by using an induction coil, using Lewis's rotary contact breaker in the primary circuit.

Results

Experiments in the Whole Animal

The results of 18 experiments which were carried out in the spinal cat may be illustrated from two of them. For ACh, KCl, and $CaCl_2$ the results were fairly consistent, but for histamine and pilocarpine they varied greatly. As shown in Fig. 1, 0.1 mg. pilocarpine nitrate was without effect on the denervated membrane or on the normal membrane, but as shown in Fig. 2 it caused both to contract, the denervated less than the normal. The effect of 40 mg. $CaCl_2$ was the same in both experiments ; both membranes contracted, the denervated more than the normal. The effect of 0.1 mg. histamine was to cause contraction of the normal membrane as shown in both Fig. 1 and Fig. 2. In Fig. 1 the denervated membrane contracted less and more slowly, while in Fig. 2 it did not contract ; it relaxed slightly. The effect of 30 mg. KCl was to cause contraction of the denervated membrane in both experiments, but it caused contraction of the normal membrane only in the second. The effect of ACh was to cause both membranes to contract, the denervated more than the normal. Very little attention was given to eserine, but in the dose of 0.3 mg. used in the experiment of Fig. 1, it had no appreciable effect.

Action of Histamine.—Since the action of histamine appeared to be of great importance in deciding how far the hypersensitivity of the denervated membrane was specific, in 18 other experiments a study of the action of histamine alone was made. The normal membrane contracted in response to histamine in 10 out of 14

experiments in spinal cats, but not in two experiments in cats under chloralose or in two experiments in cats under allobarbitone. The normal membrane contracted in each of seven experiments on spinal cats in which the adrenal glands were first excluded from the circulation. The normal membrane contracted in each of eight experiments after the cat received 8 mg. cocaine by intramuscular injection ; half of these experiments were in spinal cats and half in cats anaesthetized with chloralose or allobarbitone. The effect of cocaine is shown in Fig. 3, in which 20 μg. histamine was without effect before cocaine was given, but caused a contraction after ; in this experiment the exclusion of the adrenal glands abolished the effect of histamine.

The results with the denervated membrane were more difficult to understand. Thus only in 8 out of 18 experiments did the denervated membrane contract more than the normal membrane. In two others the denervated contracted to the same extent as the normal membrane ; in four others the denervated contracted, but less than the normal membrane ; in two others the denervated membrane did not contract although the normal membrane did, and finally in two others the denervated membrane relaxed, while the normal membrane contracted.

We have no satisfactory explanation to offer of the relaxation of the denervated membrane by histamine ; it was observed very often as a prelude to contraction. In two experiments we injected 1 mg. decamethonium to stop reflex movements of the cat, and in each of these we observed contraction of the normal membrane accompanied by apparent relaxation of the denervated membrane. We supposed that the effects of decamethonium were exerted on the external ocular

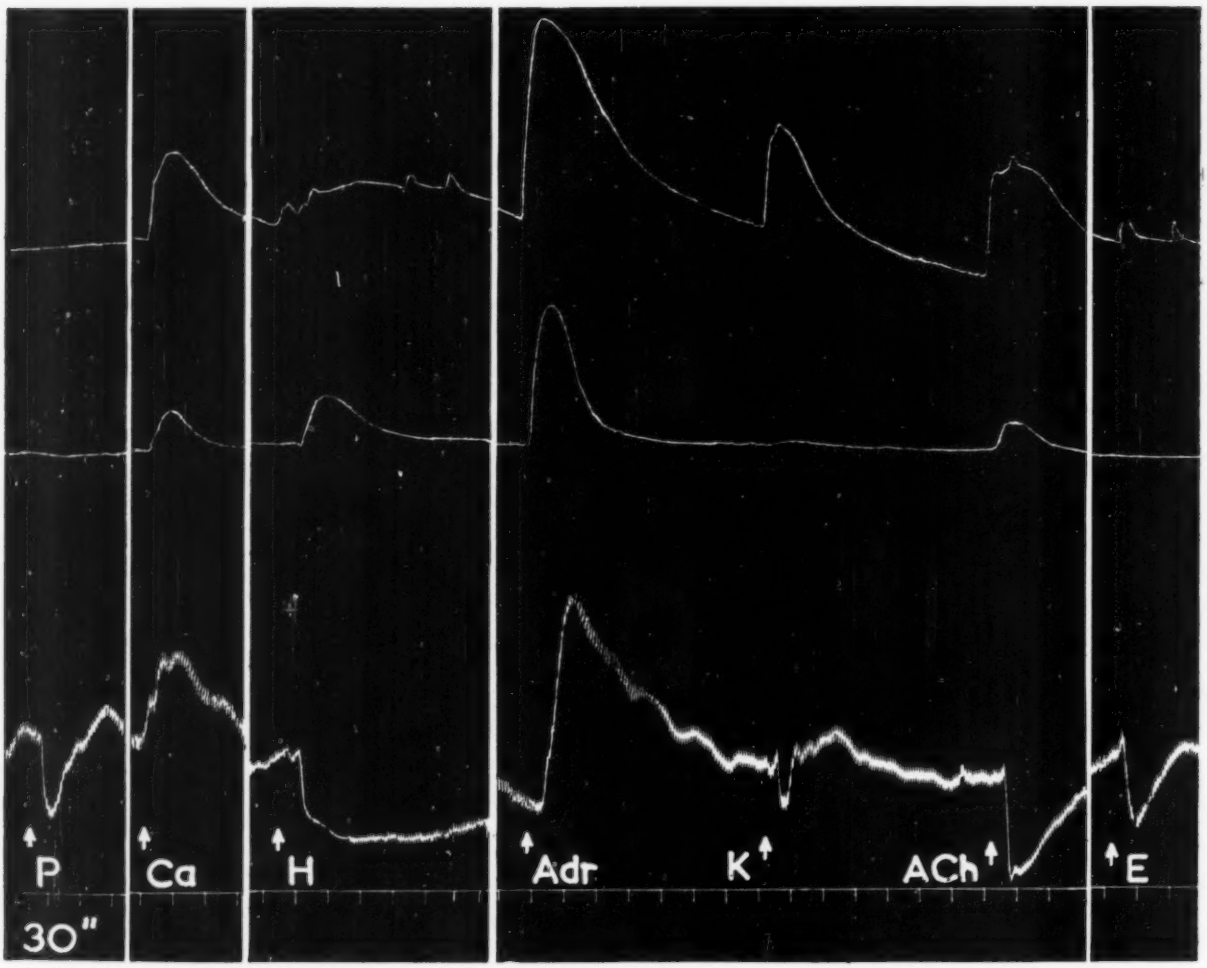

FIG. 1.—Spinal cat. Records, from above down, are: denervated nictitating membrane; normal nictitating membrane; arterial blood pressure. Intravenous injections were: P, 0.1 mg. pilocarpine nitrate; Ca, 40 mg. $CaCl_2$; H, 0.1 mg. histamine (base); Adr, 10 μg. adrenaline; K, 30 mg. KCl; ACh, 40 μg. acetylcholine; E, 0.3 mg. eserine sulphate.

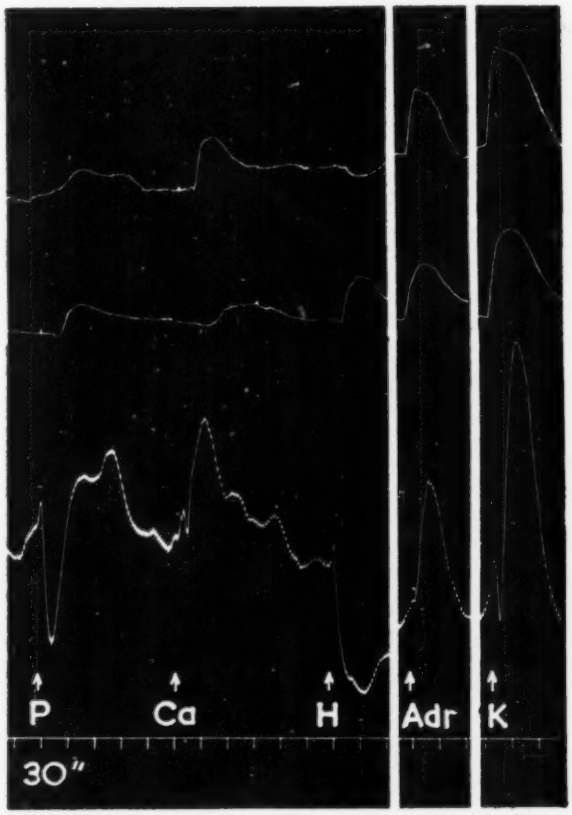

FIG. 2.—Spinal cat; records as in Fig. 1. Intravenous injections as follows: P, 0.1 mg. pilocarpine nitrate; Ca, 40 mg. $CaCl_2$; H, 0.1 mg. histamine (base); Adr, 10 μg. adrenaline; K, 30 mg. KCl.

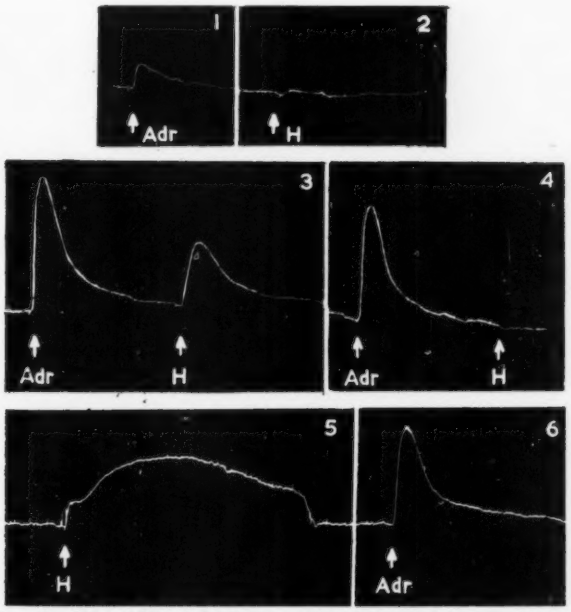

FIG. 3.—Cat under allobarbitone. Normal nictitating membrane. Contractions due to: (1) 5 μg. adrenaline. (2) 20 μg. histamine (base). (8 mg. cocaine was then injected intramuscularly.) (3) 5 μg. adrenaline and 20 μg. histamine. (The adrenals were then tied.) (4) 5 μg. adrenaline and 20 μg. histamine (40 mg./kg. cysteine was injected intravenously). (5) One hour later, 5 μg. histamine. (6) 5 μg. adrenaline.

muscles, movements of which Rosenblueth and Bard (1932) and Paton and Thompson (unpublished) have shown to affect the record of the movements of the nictitating membrane. It may be that some of the effects of histamine seen after exclusion of the adrenals are also due to action on these muscles.

In all these experiments the effect of adrenaline was observed as well as that of histamine. The effect of 5–10 μg. adrenaline was nearly always greater, usually much greater, on the denervated than the normal membrane. Moreover, the effect of adrenaline increased as the dose increased. This did not seem to be true for histamine, though different doses were tested in only four experiments. In one of these 7 μg. histamine caused the denervated membrane to contract 4 mm. (as recorded on the drum), 33 μg. caused a contraction of 6.5 mm., and 100 μg. caused a contraction also of 6.5 mm. In a second, 20 μg. histamine caused the denervated membrane to contract 7 mm.; 100 μg. caused it to contract 6 mm., and 300 μg. caused it to contract 12 mm. In a third, 20 μg., 100 μg., and 300 μg. all caused the denervated membrane to contract 3 mm. In these three experiments the denervated membrane contracted more than the normal membrane throughout. These effects are all small, not graded according to dose, and do not confirm that the membrane was usually as sensitive to histamine as to adrenaline (Gaddum and Goodwin, 1947). We tested the effect of injecting cysteine hydrochloride, 30 mg./kg., to see if we could observe on the nictitating membrane what Bein and Meier (1952) observed on the blood pressure. Five experiments were carried out in the spinal preparation, a positive result being obtained in only one; this is shown in Fig. 3. When the effect of histamine on the nictitating membrane was abolished by adrenalectomy, the injection of cysteine caused a restoration of the stimulant action one hour later; the effect was observed after the injection of 5 μg. and 20 μg. histamine.

Action of Pilocarpine.—As Fig. 1 and Fig. 2 show, the action of pilocarpine was also variable. Out of 18 experiments in which injections of pilocarpine were made, the effect on the denervated membrane was greater than that on the normal membrane in 11, it was equal to that on the normal membrane in four, and less than that on the normal membrane in three. In the 18 experiments, 28 injections were made of doses from

75 μg. to 0.5 mg. In only 9 of the 28 trials was the contraction of one of the membranes greater than 7 mm., whereas the injections of 5 μg. adrenaline in the same experiments caused a contraction greater than 7 mm. 12 out of 13 times. Thus, as with histamine, the effects of pilocarpine were small. In one experiment in which pilocarpine had no effect on the normal membranes, after the injection of cocaine it caused a contraction ; when the adrenal glands were excluded, the effect of pilocarpine was abolished.

Action of Potassium Chloride.—The action of KCl was less difficult to summarize than that of histamine or of pilocarpine. It was injected intravenously in a dose of 30 mg. in 16 experiments ; it caused a contraction of the normal membrane in 11 of these, the mean height being 8.6 mm., and a contraction of the denervated membrane in 15 of these, the mean height being 13 mm. The contraction of the denervated membrane was greater than that of the normal membrane in 11 of the 16 experiments, and about the same in the others.

Action of Calcium Chloride.—The action of $CaCl_2$ was very similar to that of KCl. A dose of 30 mg. was used in 13 experiments. The normal membrane contracted in nine of these, the mean contraction being 7.3 mm. ; the denervated membrane contracted in all experiments, the mean contraction being 14.1 mm.

Action of Acetylcholine.—ACh was studied in seven experiments only in the whole animal, for our perfusion experiments showed that it had a direct action on the nictitating membranes. When given in a dose of 40 μg. it caused contraction of the normal nictitating membrane in four out of six experiments, the mean contraction being 4 mm. ; it caused contraction of the denervated membrane in all experiments, the mean contraction being 13.8 mm.

The results so far described are summarized in Table I, which includes results for adrenaline also. They show that all substances stimulated the denervated membrane more than the normal membrane, in confirmation of earlier workers, but they also show that adrenaline produced much larger contractions in much lower doses than the rest, the effects of histamine and pilocarpine being small even when the doses used were high.

Evidence of Cholinergic Fibres.—The observations of Bacq and Fredericq (1935) were repeated in a few experiments to see if evidence of cholinergic fibres in the sympathetic supply of the nictitating membrane could be confirmed. In some experiments unipolar stimulation was applied to the ganglion itself, and eserine was applied directly to the nictitating membrane, using a solution containing 10 mg./ml. The result in one experiment is shown in Fig. 4, in which the relaxation of the membrane was slower after the application of the eserine, and then after the injection of 2 mg. atropine the height of the contraction was reduced and the relaxation was quicker.

In other experiments the cervical sympathetic chain was laid on shielded electrodes, and similar results were obtained. A positive effect of eserine was observed in four out of eight experiments, and a positive effect of atropine in seven out of

TABLE I

EFFECTS OF VARIOUS SUBSTANCES ON THE NORMAL AND ON THE DENERVATED NICTITATING MEMBRANE IN THE WHOLE ANIMAL

Substance	Dose	Normal Membrane		Denervated Membrane	
		Proportion of Expts. in which Contraction Occurred	Mean Ht. of Contraction (mm.)	Proportion of Expts. in which Contraction Occurred	Mean Ht. of Contraction (mm.)
Histamine	7–20 μg.	0/3	0	3/3	4·7
	33–50 ,,	1/2	0·8	2/2	10·3
	100 ,,	6/8	2·8	8/8	6·9
	500 ,,	1/2	2·3	2/2	7·5
Pilocarpine	75 ,,	5/7	3·3	6/7	3·6
	100 ,,	7/14	1·1	14/14	6·0
	150–200 ,,	5/5	5·8	5/5	8·4
	300 ,,	1/1	4·0	1/1	11·5
	500 ,,	2/2	2·5	2/2	16·8
KCl	30 mg.	11/16	8·6	15/16	13·1
	40 ,,	0/1	0	1/1	24
	50 ,,	0/1	0	1/1	15
$CaCl_2$	30 ,,	9/13	7·3	13/13	14·1
	40 ,,	2/2	7·5	2/2	14·3
	50 ,,	2/4	3·0	3/4	13·8
ACh	20 μg.	1/1	12 0	1/1	9·0
	40 ,,	4/6	4·0	6/6	13·8
Adrenaline	5 ,,	12/12	9·7	12/12	22
	10 ,,	10/10	15·0	10/10	29
	20 ,,	2/2	22·0	2/2	53

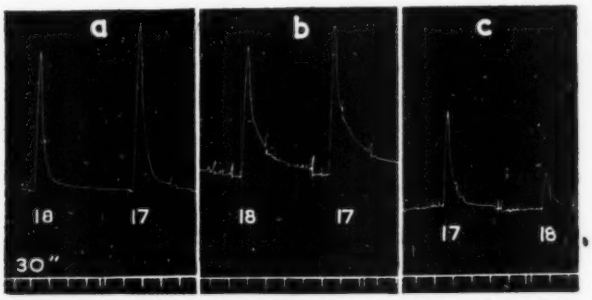

FIG. 4.—Cat under chloralose. Record of contractions of nictitating membrane when postganglionic fibres were stimulated by secondary coil (distance from primary in cm. shown under contraction). *a*, initial responses; *b*, after 1% eserine was instilled in the conjunctival sac; *c*, after 2 mg. atropine was injected intravenously. Relaxation was slower in *b*. Contractions were smaller and relaxation quicker in *c*.

seven experiments. Our results confirmed the observations of Bacq and Fredericq that in a certain proportion of cats evidence of the presence of cholinergic fibres can be obtained by this method.

Perfusion Experiments

The early perfusion experiments were carried out using Locke's solution, and were mostly unsuccessful because the tissue soon became oedematous. When, however, 25% of a 6% solution of dextran was used and the temperature was maintained at 37°, satisfactory results were obtained as shown in Figs. 5 and 6.

The general result of the perfusion experiments was that the membranes responded to the injection of adrenaline and of ACh with large contractions which increased with the dose; when the other substances were injected, in some experiments there were very small contractions, but in most experiments there were none. The results in Fig. 5 were obtained when Locke's solution only was used for perfusion. The injection of 5 μg. adrenaline caused a large contraction of the normal membrane, and a still larger contraction of the denervated membrane. The next injection was 40 mg. $CaCl_2$, which had no effect on either membrane. The next injection was 100 μg. ACh, which caused contractions similar to those caused by adrenaline. Although the preparation was capable of large responses to adrenaline and ACh, the injection of 0.2 mg. histamine produced only a tiny contraction in both membranes, and the injection of 40 mg. KCl was still less effective. In Fig. 6, when dextran–Locke was used, 25 mg. $CaCl_2$, 15 mg. KCl and 50 μg. histamine had no appreciable action, but pilocarpine caused a small contraction both in the dose of 50 μg. and of 0.5 mg. In this respect pilocarpine and histamine resembled one another and were unlike ACh and adrenaline—namely, that the injection of 10 or 20 times as much had scarcely more effect. The results of the nine best perfusion experiments are given in Table II, these being experiments in which the effects of adrenaline and ACh were reasonably large. In all, 17 perfusion experiments were carried out, and in none of these had histamine, KCl, pilocarpine, or $CaCl_2$ an effect to be compared with that of adrenaline or ACh.

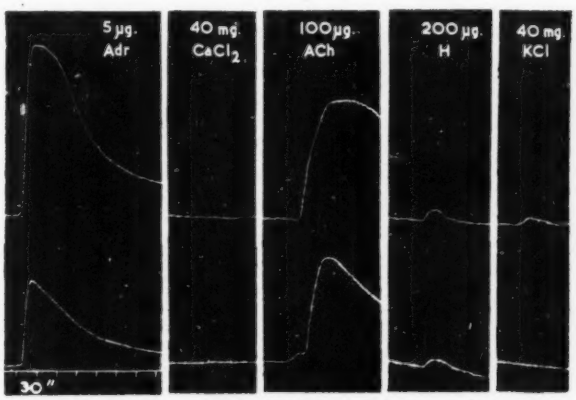

FIG. 5.—Cat's head perfused with Locke's solution. Upper record, denervated nictitating membrane; lower record, normal membrane. Successive injections of 5 μg. adrenaline; 40 mg. $CaCl_2$; 100 μg. acetylcholine; 200 μg. histamine; 40 mg. KCl.

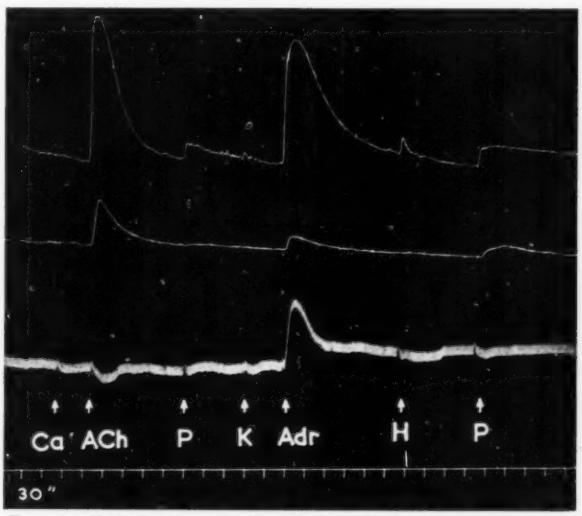

FIG. 6.—Cat's head perfused with dextran-Locke. Records as in Fig. 5, with the perfusion pressure below. Successive injections of 25 mg. $CaCl_2$; 20 μg. acetylcholine; 50 μg. pilocarpine; 15 mg. KCl; 5 μg. adrenaline; 50 μg. histamine; and 500 μg. pilocarpine.

DISCUSSION

The belief that the denervated nictitating membrane is more sensitive than the normal membrane to the action of almost any stimulant substance has been widely held since the observations of Rosenblueth (1932) and of Bacq and Rosenblueth (1934). The view is one which puts the increased sensitivity of denervated tissues almost beyond the reach of experimental investigation, since it explains the hypersensitivity as due to an increase in permeability at some undefinable point between the blood vessels and the receptors at which the chemical substance acts.

We have investigated the hypersensitivity of the denervated membrane in experiments in which the cat's head was perfused with Locke's solution containing dextran, and have found that the nictitating membranes responded to adrenaline and to ACh readily, but practically not at all to histamine, pilocarpine, $CaCl_2$, and KCl. These results appear to us to alter the situation, for they show that the

TABLE II

EFFECTS OF VARIOUS SUBSTANCES ON THE NORMAL AND ON THE DENERVATED NICTITATING MEMBRANE IN THE PERFUSED HEAD

(Figures are heights of contraction in mm. NM = normal membrane. DM = denervated membrane)

Exp.	Adrenaline			Histamine			Pilocarpine			KCl			$CaCl_2$			Acetylcholine		
	Dose μg.	NM	DM	Dose μg.	NM	DM	Dose μg.	NM	DM	Dose mg.	NM	DM	Dose mg.	NM	DM	Dose μg.	NM	DM
1	5	4	39	50	0	5	50	0	5	15	0	2	30	0	0	30	13	42
	—	—	—	500	1	5	500	4	6	—	—	—	—	—	—	—	—	—
2	5	3	40	50	0	1	50	0	0	15	0	0	15	0	0	25	6	7
	—	—	—	—	—	—	1 mg.	3	2	—	—	—	100	0	0	—	—	—
3	5	2·5	12	50	0	0	50	2	0	15	0	0	—	—	—	20	4	6
	—	—	—	200	0	1	250	0	2	60	0	1	—	—	—	50	3	9
4	5	2	19	50	0	0	50	0	0	15	0	0	15	0	0	20	1	9
	—	—	—	100	1	1	—	—	—	—	—	—	—	—	—	—	—	—
5	5	2	8	50	0	0	50	1	5	30	0	3	30	0	0	20	4	13
	—	—	—	100	0	2	—	—	—	—	—	—	—	—	—	—	—	—
6	2·5	2	10	50	0	0	50	0	0	15	0	0	—	—	—	50	3	3
				200	0	2	500	0	0	30	0	0	—	—	—	—	—	—
7	5	2	26	50	0	3	100	0	0	15	0	0	15	0	0	100	4	20
8	2	6	13	200	3	6	—	—	—	40	0	0	40	0	0	40	8	11
9	5	25	50	40	3	1	—	—	—	20	0	0	40	0	1	100	30	37
	—	—	—	200	3	3	—	—	—	40	0	2	—	—	—	—	—	—

action of histamine, pilocarpine, $CaCl_2$, and KCl which is seen in the body is almost certainly an indirect action due to the release of an adrenaline-like substance. There have been several observations previously made which ought to have suggested this. In the first place the effect of histamine, and of some others of these substances, is augmented by cocaine. There are no other effects of histamine which are augmented by cocaine. Augmentation by cocaine is, however, well recognized to occur with noradrenaline and adrenaline. In the second place, Ross (1936) showed that the contractions of the denervated nictitating membrane caused by adrenaline, $CaCl_2$, and KCl were reduced by 933F and yohimbine; the contraction caused by ACh was much less reduced. These anti-adrenaline substances do not reduce other effects of $CaCl_2$ and KCl.

That many substances release adrenaline and noradrenaline from the adrenal glands has long been known; for this reason previous observers have excluded the adrenals. We have found more than once that, after this exclusion, histamine and pilocarpine have no action on the membranes even in the cat treated with cocaine. Any direct action they may have must therefore be very variable, and unlike the regular action of adrenaline. Bein and Meier (1952) have shown that the pressor action of histamine, seen to follow the initial fall in the spinal cat, disappears after adrenalectomy, but can be restored by the injection of L-cysteine. We have observed in one experiment that, when the effect of histamine on the normal nictitating membrane was abolished by adrenalectomy, the injection of L-cysteine restored it. Now, unless we suppose that L-cysteine "sensitizes" the nictitating membrane to histamine, we seem to have evidence in this one case that histamine was acting by liberating some other substance. Since the adrenal glands were excluded, the release must have been from some other tissue. In view of the fact, demonstrated by Gaddum and Goodwin, that some doses of histamine have less effect on the denervated membrane than on the normal membrane, which we have also observed, it seems likely that the hypothetical substance released by histamine is neither adrenaline nor noradrenaline.

While putting these points mainly as suggestions for further inquiry, our evidence showed that adrenaline, ACh, and noradrenaline were the only substances to which the denervated nictitating membrane responded freely when the head of the cat was perfused with Locke's solution. It removed a serious obstacle to the view that the hypersensitivity of the denervated membrane to the substances which affect it directly can be explained by the fall in the enzymes.

The observation that histamine had in some perfusion experiments a very small effect on the nictitating membrane seems best accounted for by the fact that histamine has some adrenaline-like actions, as, for example, on the coronary vessels of the cat and dog and on the uterus of the rat and mouse, so that it may have some slight

power of attaching itself to receptors for adrenaline. Similarly, pilocarpine is a substance having properties similar to those of histamine on the one hand (Burn, 1925), and to those of ACh on the other. Hence pilocarpine may also be expected to have some slight power of stimulating receptors for adrenaline or for ACh. The fact that slight effects on the denervated membrane due to histamine, pilocarpine, or KCl were not seen on the normal membrane can be interpreted by the loss of enzyme in the denervated tissue. If these substances have some very small power of combining with adrenaline or ACh receptors, presumably they have some very small power of combining with the sites on the enzymes to which adrenaline or ACh normally become attached. In the denervated tissue, where the enzyme receptors have been shown to be fewer, more molecules of histamine, pilocarpine, or KCl will combine with the receptors and cause some stimulation.

Summary

1. The denervated nictitating membrane of the cat has been shown by others to contract more than the normal membrane in response to injections of KCl, $CaCl_2$, ACh, pilocarpine, histamine, and eserine ; it has further been stated that the effects of these substances on the nictitating membranes are not dependent on the presence of the adrenal glands.

2. We have made observations by perfusing the cat's head with Locke's solution containing dextran, and have found that the only substances to which the denervated and normal nictitating membranes readily respond are adrenaline and ACh. We have never observed a response to $CaCl_2$, and responses to KCl, histamine, and pilocarpine have always been very small in the few experiments in which they were present, no matter how large the amount injected.

3. We have confirmed the evidence of Bacq and Fredericq that there is a small proportion of cholinergic fibres in the sympathetic supply of the nictitating membrane.

4. We conclude that the effects of KCl, $CaCl_2$, histamine, and pilocarpine in the whole animal after exclusion of the adrenals are indirect effects due to the liberation of an adrenaline-like substance from some other organ. These effects are in any case sometimes absent and usually small.

5. Since denervation of the nictitating membrane has been shown to cause a fall in amine oxidase and in true cholinesterase, the hypersensitivity of the membrane to adrenaline and ACh can be explained by the decline in the amount of these enzymes.

In the early part of this work we had the pleasure of the collaboration of Dr. A. D. Welch, Yale University, who was a Fulbright Visiting Professor.

One of us (U. T.) has carried out the work while holding a British Council Scholarship.

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OBSERVATIONS ON THE CHARACTER OF MERCURIAL DIURESIS

BY

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The very profound effect of mercurial diuretics on the excretion of sodium, chloride and water has been variously ascribed to an action on the proximal (Mudge, Foulks, and Gilman, 1949; Weston, Grossman, and Leiter, 1951) and the distal renal tubule (Duggan and Pitts, 1950). Comparatively little attention has been paid to the other constituents of urine. This paper describes the effect of mersalyl on the excretion of potassium, phosphate, hydrogen ion, ammonia, bicarbonate, uric acid, and creatinine in normal human subjects. Too little is known of the mechanisms governing the excretion of these substances to allow the data to be fitted into any single hypothesis. Nevertheless, the data seem worth recording, as they will have to be taken into account in any final hypothesis concerning the site and mode of action of the drug.

METHODS

The subjects were normal male medical students or doctors. They came to the laboratory in the morning, having taken nothing by mouth since the previous evening. The experiments were begun as near 9.30 a.m. as possible. Urine was collected at 30 min. intervals by voluntary micturition, without a catheter; the collection periods were usually reduced to 15 min. at the peak of the diuresis.

The mercurial diuretic used was Mersalyl, obtained as the solid from British Drug Houses Ltd. The dose was always 0.2 g., dissolved in 20 ml. sterile distilled water and injected intravenously as soon as possible after solution. The injection was given slowly over a period of 10 min. to avoid possible toxic effects on the heart resulting from a momentarily excessive blood level of the drug. No theophylline was given with these injections.

During the experiments the subjects sat in chairs or reclined on couches; their activity was restricted to that necessary for passing urine. They took nothing by mouth, except as stated in the text. Smoking was prohibited.

The chemical methods used were as follows: Potassium, direct reading flame photometer; inorganic phosphate, Fiske and Subbarow (1925); ammonia, Conway and O'Malley (1942); *p*H, glass electrode; uric acid, Benedict and Franke (1922); creatinine, Folin (1914); carbon dioxide, Van Slyke and Neill (1924).

RESULTS

Potassium.—Fig. 1 illustrates a typical experiment. After five preliminary 30 min. periods, 0.2 g. of mersalyl was injected intravenously; the concentration and rate of excretion of urinary

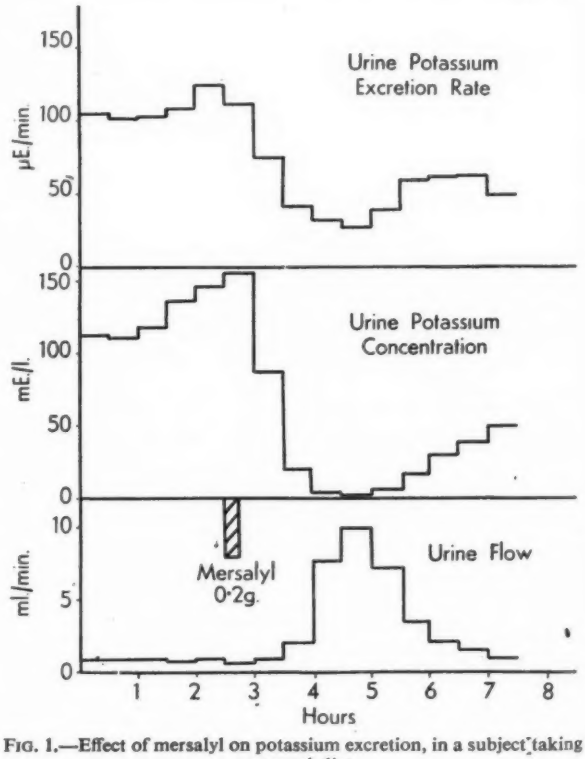

FIG. 1.—Effect of mersalyl on potassium excretion, in a subject taking a normal diet.

potassium fell conspicuously. Table I shows the results of 19 similar experiments in terms of urinary concentration and urinary excretion rate, during the initial control period and at the peak of diuresis. In four of these experiments (marked with an asterisk), the subjects drank 0.9% NaCl solution throughout the experiment at a rate of 200 ml./hr. The object of this was to increase the subjects' reserves of sodium and chloride, in the hope that a more profuse diuresis would result when mersalyl was given. Control experiments without mersalyl had previously shown that drinking saline in this way caused no significant increase in urine flow even if continued for some hours. No increase in diuresis following mersalyl resulted from this procedure ; the other results showed no obvious difference from those of the experiments without saline. These four experiments have therefore been grouped with the rest in Table I.

TABLE I

EFFECT OF MERSALYL ON POTASSIUM EXCRETION

Subject	Diet	Urine Potassium Concentration (m.equiv./l.)		Plasma Potassium (m. equiv./l.)	Urine Potassium Excretion Rate (μ.equiv./min.)	
		Initial	Peak		Initial	Peak
R.A.D. ..	N	101	4·0	4·7	91	35
R.E.W. ..	N	119	11·9	4·3	75	44
I.C.S.N. ..	N	125	2·7	4·2	108	27
E.R.D. ..	N	89	6·9	4·0	39	66
B.C.* ..	N	98	1·07	4·4	84	14
R.B.P. ..	N	116	4·5	4·3	150	63
J.S.B.* ..	N	54	10·2	4·6	90	50
T.C.W.* ..	N	85	5·6	4·0	79	48
J.E.G.* ..	N	107	17·8	4·7	103	85
M.B.T. ..	N	93	8·1	4·4	120	55
P.S. ..	N	115	2·2	3·7	111	16
P.A.B. ..	N	179	3·9	3·9	115	47
T.O.W. ..	SP	72	9·7	4·3	30	77
I.C.S.N. ..	SP	62	11·7	4·2	32	97
P.A.B. ..	SP	134	13·2	3·8	63	130
B.C. ..	SP	109	11·9	3·9	52	69
M.H. ..	SP	127	9·1	3·6	87	56
W.R.C. ..	SP	9·1	11·4	3·6	19	192
E.R.D. ..	SP	71·3	19	—	34	70

* Indicates that the subject drank 0·9% NaCl solution, 200 ml./hr., throughout the experiment. N = normal diet. SP = salt poor diet.

In seven of the experiments (marked " SP ") the subjects ate a diet supplying some 0.5 g. of sodium and 1 g. of chloride daily for two or three days before the experiment.

Of the twelve experiments on subjects taking a normal diet, all except one showed a fall both in the concentration and in the excretion rate of potassium. On the other hand, when a salt-poor diet was taken, mersalyl caused a rise in the excretion rate in six out of seven experiments, although the fall in concentration occurred as in the other experiments. Fig. 2 illustrates a result of this type. Although the concentration of potassium in the urine fell in every experiment, there

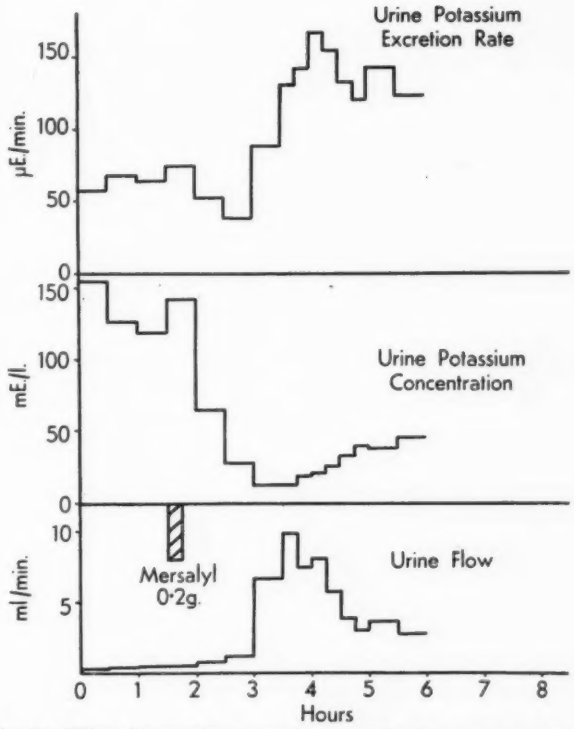

FIG. 2.—Effect of mersalyl on potassium excretion, in a subject taking a diet poor in sodium and chloride. Note the rise in excretion rate, in contrast to the fall seen in Fig. 1.

seemed no real tendency—as had been found with sodium (Dale and Sanderson, to be published)—for the urinary potassium level to approximate that of plasma. Thus, in most of the experiments in Table I, the urinary potassium at the peak of the diuresis greatly exceeded the plasma level ; but in three it was much lower.

Inorganic Phosphate.—The results of eleven experiments are shown in Table II. There was a conspicuous fall in the urinary concentration in

TABLE II

EFFECT OF MERSALYL ON THE EXCRETION OF INORGANIC PHOSPHATE

Subject	Urine Inorganic Phosphate Concentration (m.equiv./l.)		Plasma Inorganic Phosphate (m. equiv./l.)	Urine Inorganic Phosphate Excretion rate (μ.equiv./min.)	
	Initial	Peak		Initial	Peak
R.A.D. ..	10·8	0·35	1·53	9·65	3·1
R.E.W. ..	14·9	5·45	1·20	9·5	20·3
I.C.S.N. ..	14·5	1·84	1·77	12·5	18·4
E.R.D. ..	6·4	0·88	1·00	2·75	8·4
B.C.* ..	7·5	1·19	1·19	6·5	15·3
R.B.P. ..	12·1	0·72	1·38	15·3	10·1
J.S.B.* ..	11·9	3·94	1·02	19·6	19·4
T.C.W.* ..	9·8	0·83	1·43	9·1	7·1
J.E.G.* ..	6·7	2·10	—	6·1	10·5
M.B.T. ..	11·7	2·59	—	15·1	17·5
P.S.	7·8	0·67	—	7·5	4·7

* Subject drank 0·9% NaCl.

each. The effect on the excretion rate was variable; there was a rise in six experiments and a fall in five. Weston, Grossman, and Leiter (1951), using thiomerin, concluded that the usual effect was a rise in the excretion rate. In interpreting their results it must be remembered that there is a diurnal change in the rate of phosphate excretion in the normal subject, such that the rate in the afternoon tends to be higher than that in the morning. In Table III are shown the results of ten control experiments, in which the subjects came with the same preparation as in the mersalyl experiments, and produced urine specimens under

TABLE III

DIURNAL CHANGE OF URINARY INORGANIC PHOSPHATE EXCRETION IN NORMAL SUBJECTS WITHOUT MERSALYL INJECTION

Subject	Initial phosphate excretion rate (μ. equiv./min.)	Time	Highest subsequent phosphate excretion rate (μ. equiv./min.)	Time
R.E.W. ..	12·9	9.40 a.m.	21·4	4·40 p.m.
R.A.D. ..	6·3	11.00 ,,	13·2	4.00 ,,
R.B.P. ..	6·2	10.10 ,,	15·6	5.10 ,,
I.C.S.N. ..	10·3	10 00 ,,	25·6	5.00 ,,
M.B.T. ..	14·5	10.10 ,,	22·1	4.10 ,,
P.S. ..	23·6	10.15 ,,	15·8	4.15 ,,
B.C.* ..	0·7	10.10 ,,	12·7	4.40 ,,
T.C.W.*	11·0	10.10 ,,	33·2	4 40 ,,
J.E.G.* ..	11·5	10.10 ,,	21·6	5.10 ,,
J.S.B.* ..	22·2	10.30 ,,	26·5	2.30 ,,

* Subject drank 0·9% NaCl

the same conditions, but had no mersalyl. The rate of phosphate excretion in the first timed collection of urine, and the highest subsequent excretion rate, are recorded, together with the times of passing the urine specimen concerned. In all except one subject, the excretion rate rose to a higher level in the afternoon, often to twice or three times, and in one individual to nearly twenty times, the morning rate. Weston *et al.* do not say at what time of day their experiments were done; Figs. 1 and 2 show, however, that an experiment carried on until the rate of urine flow is returning to the preliminary figures is likely to last some six or seven hours. If, as seems likely, Weston and co-workers began their experiments between 9 and 11 a.m., it appears doubtful whether any rise in phosphate excretion they observed can be ascribed to the injection of thiomerin. In fact, it could be argued, from a comparison of Tables II and III, that the action of mersalyl as observed by us was occasionally to convert the normal rise in phosphate excretion into a fall. It is perhaps safest to conclude that mersalyl has no constant effect in this respect.

Hydrogen Ion.—Fig. 3 shows the effect of mersalyl on urinary *p*H. The two lines are the averaged results of twelve experiments with

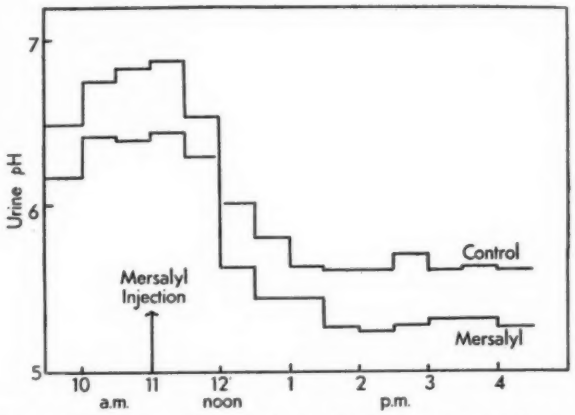

FIG. 3.—Effect of mersalyl on urinary *p*H. The control experiments show a trend towards increased acidity during the course of the day, which is intensified when mersalyl is given.

mersalyl and of eleven control experiments without mersalyl. The time scale is that of the control experiments; in the mersalyl experiments the injection has been taken as the reference point, and the periods have been averaged by arranging them according to their time before or after this. The actual time of injection ranged between 10 a.m. and 12 noon with a mean of 11.07; accordingly, for purposes of comparison, the mersalyl series has been represented with the injection taking place at 11 o'clock. The figure shows the trend towards a more acid urine (which occurs in any normal subject) during the latter part of the day, and the more marked change in *p*H which occurs when mersalyl is given.

Ammonia.—Changes in concentration and excretion rate of urinary ammonia were observed in seven experiments, in three of which the subject took 12 g. NH_4Cl by mouth daily for two days prior to the experiment, with a dose of 2 g. at about 8 a.m. on the morning of the experiment. The results are shown in Table IV. The effect of mersalyl was always to diminish the concentration of urinary ammonia; the effect on rate of excretion of ammonia, however, varied, depending on whether or not the subject had been taking NH_4Cl. The ammonia excretion of those subjects who had not taken NH_4Cl was initially comparatively low (mean of four experiments, 15.2 mE./min.), and rose during the diuresis to a mean value of 46.9 mE./min., whereas in the subjects who had taken NH_4Cl the ammonia excretion, initially high (mean of three experiments, 79.2 mE./min.)

TABLE IV

EFFECT OF MERSALYL ON THE EXCRETION OF AMMONIA

Subject	Date	Concentration (m.equiv./l.) Initial	Concentration (m.equiv./l.) Peak	Excretion (μ equiv./min.) Initial	Excretion (μ equiv./min.) Peak
J.E.G. ..	9.8.51	31·8	11·3	31·4	56·5
M.B.T. ..	24.9.51	6·5	4·65	8·2	31·6
P.S. ..	18.10.51	9·0	7·5	8·2	52·0
P.A.B. ..	8.11.51	20·2	3·9	12·9	47·6
J.G.O.* ..	11.2.52	66·0	7·1	78·5	48·3
T.O.H.* ..	13.2.52	53·1	4·9	83·5	59·1
R.M.L.* ..	21.2.52	75·7	4·4	75·7	59·9

* Subject took NH_4Cl before the experiment.

fell after mersalyl to a mean value, at the peak of diuresis, of 55.8 mE./min. The urinary *p*H values in these latter experiments (which were not included in the compilation of Fig. 3) showed the usual trend towards increased acidity (see Fig. 5), but the initial values were much lower—between *p*H 4.7 and *p*H 5—in all three subjects (compare Fig. 3), and the subsequent change towards acidity extended only as far as *p*H 4.3–4.6, which is probably close to the limiting value for the human kidney (Pitts, 1948). Since we performed no control experiments with NH_4Cl, we are unable to say whether this increase in acidity was due to mersalyl or to the normal diurnal variation; but, in any case, we have in these experiments the unusual situation of an increase in acidity associated with a fall in ammonia output.

Bicarbonate.—From the total CO_2 content of the urine and its *p*H, the bicarbonate concentration was calculated. Fig. 4 shows the changes in *p*H and excretion rate of ammonia and bicarbonate induced by mersalyl in a subject without preliminary medication: Fig. 5 illustrates the same changes in a subject who had been taking NH_4Cl as detailed above. Fig. 4 shows a considerable fall in *p*H and the expected changes in ammonia and bicarbonate output—a rise and a fall respectively. Fig. 5 shows the paradoxical fall in ammonia output noted above, together with a rise in bicarbonate output. It should be noted that the amounts of bicarbonate involved in Fig. 5 are extremely small, so that if plotted on the same scale as Fig. 4 most of the lines would coincide with the zero line. Similar changes in bicarbonate output were found in the other two experiments with mersalyl and NH_4Cl, initial values of 0.061 μE./min. and 0.088 μE./min. rising to 0.34 μE./min. and 1.165 μE./min. at the peak of diuresis. Although the amounts involved are so small, the results are in sharp contrast with the experiments in which NH_4Cl was not given. An attempt to study the effect of mersalyl on bicarbonate excretion in subjects made alkalotic by the

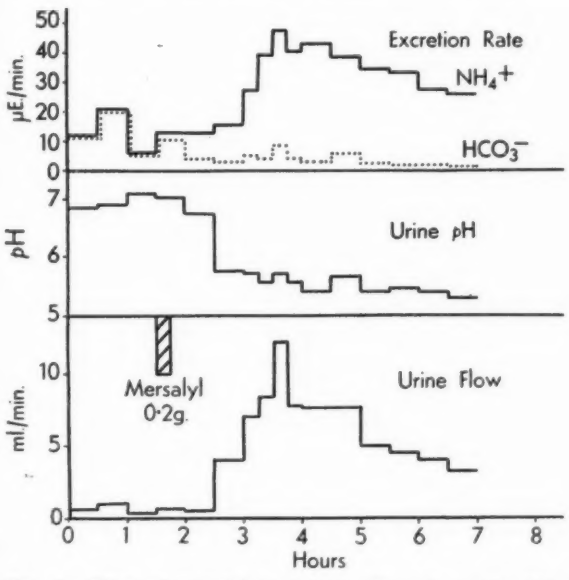

FIG. 4.—Effect of mersalyl on urinary *p*H, and on excretion of ammonia and bicarbonate, in a normal subject.

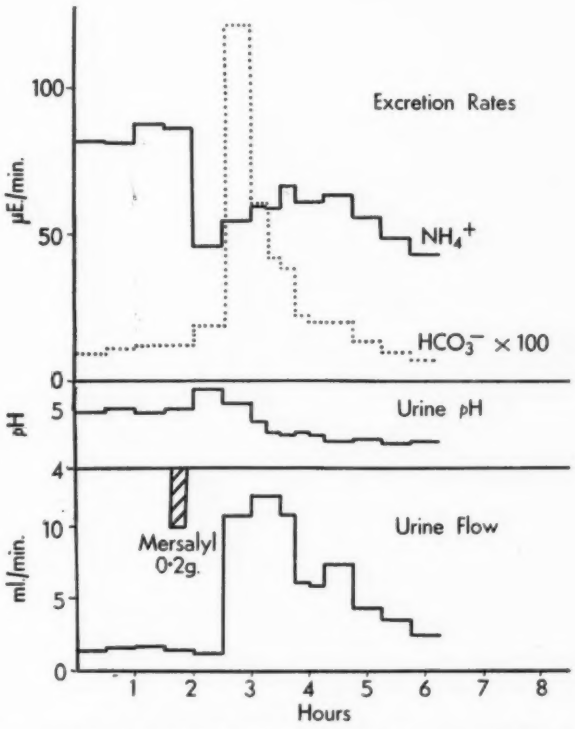

FIG. 5.—Effect of mersalyl on urinary *p*H, and on excretion of ammonia and bicarbonate, in a subject made acidotic by ingestion of NH_4Cl. Note the fall in ammonia output and rise in bicarbonate output, in spite of the usual increase in urinary acidity.

ingestion of $NaHCO_3$ was partly frustrated by the inhibitory effect of this regime on a mercurial diuresis. Three subjects took 8 g. $NaHCO_3$ three times a day for two days before the experiment; two also took 8 g. on the morning of the experiment. In these two subjects, whose plasma CO_2 levels were 36.6 and 33.3 mE./l., 0.2 g. mersalyl given intravenously produced no diuresis whatever; in the third subject, who had no bicarbonate on the morning of the experiment, and whose plasma CO_2 was 31.0 mE./l., urine flow increased from a pre-injection level of 1.35 ml./min. to a peak of 5.73 ml./min. In this subject the pre-injection rate of bicarbonate excretion was 60 μE./min. (cf. Fig. 4), and with the establishment of diuresis this fell to about 1 μE./min. and remained at this level for the rest of the experiment.

In the other two subjects, the initial rates of bicarbonate excretion were much higher (627 and 327 μE./min.) and, although no diuresis occurred, bicarbonate excretion fell, abruptly in the first subject and more gradually in the second, finally reaching rates of 26 and 54 μE./min. respectively.

Uric Acid.—The effect on uric acid excretion was studied in three experiments, with closely similar results in all three. Fig. 6 shows the results of one of these. The considerable increase in uric acid elimination shown here is a well-known feature of the action of mersalyl; similar results have been reported for thiomerin by Grossman, Weston, Edelman, and Leiter (1950). Fig. 6 is included simply to draw attention to an interesting feature of the uric acid response—namely, its relationship in time to the injection of mersalyl. Whereas the rates of water, sodium, and chloride excretion commonly show no change for a full hour after the injection, the concentration and excretion rate of uric acid show a marked increase in the first 30 minutes after the injection. This was also observed in both of our other experiments. It was commented upon by Grossman and colleagues.

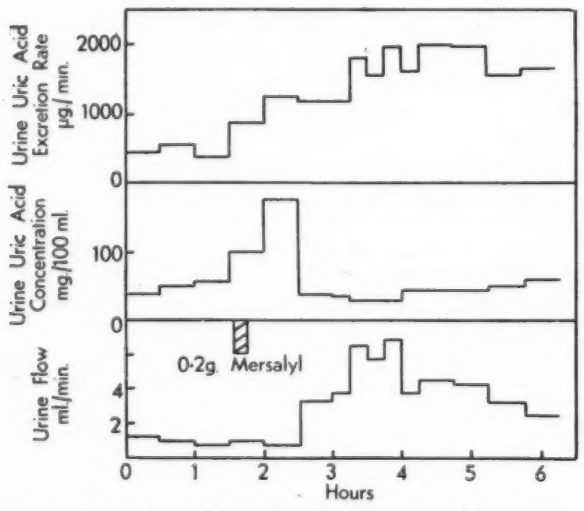

FIG. 6.—Effect of mersalyl on uric acid excretion. Note the immediate effect on uric acid, and the delayed effect on water excretion.

Creatinine.—Fig. 7 shows the mean rate of creatinine excretion in 12 experiments with mersalyl and 11 control experiments. For purposes of comparison, the same method has been used as in the compilation of Fig. 3, that is, the mersalyl experiments are represented with the injection taking place at 11 a.m., although the actual time of injection varied between 10 a.m. and 12 noon. The lines representing control and mersalyl series lie close together except during the last two periods before, and the second, third, and fourth periods after, the injection. When the significance of these differences is tested, "P" for both periods before, and for the fourth period after the injection, is greater than 0.1; for the second and third periods after the injection "P" lies between 0.05 and 0.02, and 0.01 and 0.001, respectively. During these periods there is a real decrease in creatinine excretion.

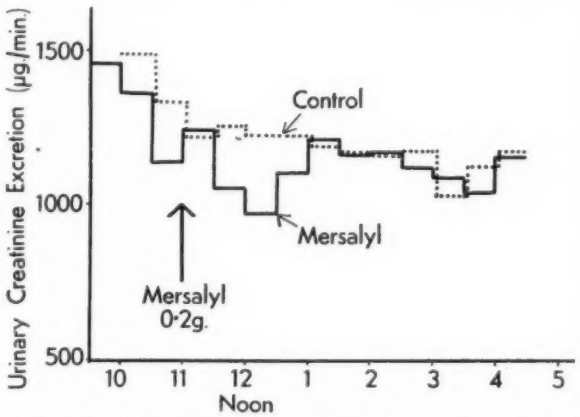

FIG. 7.—Creatinine excretion in control experiments (. . . . .) and mersalyl experiments (———).

DISCUSSION

These results show that the effect of mersalyl on the kidney extends to a wide range of substances in addition to water, sodium, and chloride. Elsewhere (Dale and Sanderson, to be published) we have presented data suggesting that the action of mersalyl on the excretion of water, sodium, and chloride can best be explained as an effect on the distal tubule. The present results, while not neces-

sarily supporting this hypothesis, do not conflict with it. Their interpretation is in some cases relatively straightforward ; in others it will have to await further elucidation of the exact events of the excretory process.

Of the organic solutes studied, creatinine is probably excreted by a combination of filtration and tubular excretion (Smith, 1951) ; the exact significance of the diminished creatinine excretion rate between 30 and 90 min. after the injection of mersalyl is therefore doubtful. However, decreases in inulin clearance after injection of other mercurial diuretics have been observed by Capps, Wiggins, Axelrod, and Pitts (1952) and after injection of mersalyl by ourselves, and it seems likely that the present results are due to transient decreases in glomerular filtration rate. The means whereby this effect is produced is at present obscure.

The behaviour of uric acid suggests the possibility of more than one mercury sensitive mechanism in the tubules—for example, a mechanism for reabsorbing uric acid which is inhibited directly and immediately by mersalyl, and one for reabsorbing water, sodium, and chloride which is inhibited only after a prolonged delay, perhaps as a result of some indirect effect of the drug. The injection of mersalyl and the beginning of the diuresis are usually separated by some 1½ to 2 hr., and the events taking place in the kidney during this time are obviously of the greatest interest and worthy of closer study.

None of the inorganic substances studied undergo such striking changes in excretion as do sodium and chloride, and very much less is known about the sites of their reabsorption. Nevertheless, the clear-cut effects found with some ions constitute a challenge to our ignorance.

The changes in potassium may be related primarily to the excretion rate prevailing before the injection of mersalyl rather than to the excretion rate of sodium or chloride, since Berliner (personal communication) has also observed that mercurials tend to lower a high excretion, and to raise a low excretion, of potassium. There is some evidence (Platt, 1950 ; Berliner and Kennedy, 1948) that two systems exist in the tubules for the regulation of potassium excretion, one being capable of active excretion ; our data would be compatible with the idea that a relatively fixed quantity of potassium was reabsorbed by the first mechanism, and that the second mechanism, located more distally and capable of inhibition by mersalyl, added to or subtracted from the remaining potassium by reabsorption or secretion as required.

Our results indicate that mersalyl causes a fall in the *p*H of the urine. It might be argued from this that the drug's action, which is almost certainly inhibitory, is unlikely to take place in the distal tubule, since this is commonly supposed to be the site of acidification of urine in mammals. However, it should be noted that this idea is based on amphibia (Richards, 1929 ; Montgomery and Pierce, 1937) and birds (Chambers and Kempton, 1933) ; the relevant observations on mammals have not been made, and the hypothesis that acidification occurs in mammalian distal tubules remains without experimental support. Walker, Bott, Oliver, and McDowell (1941) found that urine from the proximal half of the proximal tubule in rats and guinea-pigs was isotonic with plasma, and contained the same concentration of sodium, but about 40% more chloride. They pointed out that this implied the proximal reabsorption of some anion other than chloride, and suggested that this might be bicarbonate. Calculation shows that, when allowance is made for the Donnan equilibrium, the observed ratios could be accounted for by an almost complete reabsorption of bicarbonate. This is more than just a theoretical possibility ; McMaster and Elman (1928), investigating the intracellular *p*H of various tissues in rats and guinea-pigs by the intra-vital administration of erythrolitmin, found the indicator in its acid form in all the cells of the renal tubule, except those of the upper end of the proximal tubule, where it was in the alkaline form. The extent of this alkaline region could be varied by feeding acids or alkalis : acids caused it to extend a lesser, and alkalis a greater, distance from the glomerulus.

There would seem to be some evidence, therefore, for the reabsorption of considerable amounts of bicarbonate in the proximal tubules of mammals. (It may be noted at this point that no such evidence exists for amphibia ; the proximal tubule chloride U/P ratio averages 1.1, which is what would be expected in glomerular filtrate if the Donnan factor were 0.95.) It is very unlikely that any bicarbonate reabsorbed in this way would be accompanied by equivalent amounts of CO_2, since the latter is highly diffusible and apparently passes with ease across cell boundaries. It may be presumed, therefore, that the tubular fluid has a partial pressure of CO_2 of the same order as that of the blood, in which event the extensive reabsorption of bicarbonate would cause the proximal tubular fluid to become acid. Although there is no direct experimental proof of this proximal acidification, it is at least compatible with such facts as are known and would explain the increased acidity

of urine caused by mersalyl if the latter acts, as we believe, on the distal tubule.

In normal or mildly alkalotic subjects, the fall in urinary *p*H caused by mersalyl is accompanied by the expected rise and fall respectively in the excretion of ammonia and bicarbonate. In the presence of acidosis, the urinary *p*H falls, probably to a limiting value, but the changes in ammonia and bicarbonate excretion are reversed. In gross alkalosis no diuresis occurs; there is a fall in bicarbonate excretion, but whether this is due to the action of the drug or to the normal diurnal rhythym is not certain.

Too little is known at present about the mechanisms governing the excretion of hydrogen ion, ammonia, and bicarbonate for the interpretation of these data. The most suggestive observations are those made on the acidotic subjects, where the linkage between urinary *p*H and ammonia and bicarbonate excretion, supposed by Pitts (1948) to depend on simple physico-chemical mechanisms, appears to be reversed.

The abolition of mercurial diuresis in patients made alkalotic by ingestion of $NaHCO_3$ was first observed by Saxl and Erlsbacher (1929) and later by Ethridge, Myers, and Fulton (1936). An important related problem is that of resistance to mercurials in patients with cardiac failure, many of whom are alkalotic. The response can be improved by giving NH_4Cl, and it seems probable that the action of these drugs depends in some way on the *p*H of the plasma, or its chloride content. It is conceivable, for example, that the organic molecule is pharmacologically inert, and that release from it of an active constituent—possibly inorganic mercury—is dependent on an acid reaction, either in the plasma or perhaps in the renal tubules. The problem is obviously capable of further experimental study.

It will be clear that the time is not yet ripe for any single hypothesis to link up these data. What is abundantly clear is that the action of mersalyl, if studied in detail, reveals unexpected features which may well provide a key to the further understanding of the complexities of tubular function. Four points, in particular, emerge from the present results as worthy of closer attention: the changes in potassium secretion; the delay in onset of diuresis; the reversal of the usual changes in ammonia and bicarbonate excretion in acidosis; and the abolition of the diuresis in alkalosis. Study of phenomena such as these, no less than of more physiological changes, will help lead to the final goal of an integrated theory of renal behaviour.

Summary

1. The effect of mersalyl (0.2 g. intravenously) on the excretion of potassium, phosphate, hydrogen ion, ammonia, bicarbonate, uric acid and creatinine in normal human subjects has been studied.

2. In subjects taking a normal diet, mersalyl causes a fall in potassium excretion; in subjects taking a diet poor in salt, it causes a rise.

3. Mersalyl has no constant effect on the excretion of inorganic phosphate.

4. Mersalyl causes an increase in urinary acidity.

5. In subjects in normal acid-base balance, mersalyl causes a rise in ammonia excretion and a fall in bicarbonate excretion. In subjects made acidotic by the ingestion of ammonium chloride, these effects are reversed, although the usual fall in urinary *p*H occurs.

6. The increase in uric acid excretion caused by mersalyl occurs within 30 minutes of the injection, whereas the increase in water excretion is usually delayed until more than 60 minutes after injection.

7. Mersalyl causes a transient fall in the rate of creatinine excretion between 30 and 90 minutes after injection.

8. The implications of these findings are discussed.

Our thanks are due to Mr. F. Diggins and Miss V. Holloway for technical assistance; to Miss M. Furnivall for devising, and Mrs. Rhodes and Miss Luget for preparing, the salt-poor diets; to our subjects for their co-operation; and to Professor G. W. Pickering for valuable advice and criticism.

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A COMPARISON OF THE PERIPHERAL PARASYMPATHOLYTIC AND AUTONOMIC GANGLION BLOCKING ACTIVITIES OF METHANTHELINE ("BANTHINE") AND PROPANTHELINE ("PRO-BANTHINE") WITH ATROPINE AND HEXAMETHONIUM

BY

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Originally prepared by Burtner and Cusic (1943), methantheline (β-diethylaminoethyl xanthene-9-carboxylate methobromide) has been studied pharmacologically by several workers, notably Hambourger, Cook, Winbury, and Freese (1950), Lehman and Knoeffel (1944), Chittum, Longino, Metcalf, and Grimson (1949), Longino, Grimson, Chittum, and Metcalf (1950), and Lyons, Reeves, and Grimson (1950). Their work shows that with progressively increasing doses and concentrations of methantheline there appears first an atropine-like action, followed, at a higher dosage range, by autonomic ganglion blockade and finally, in relatively high doses, by a curare-like action.

Propantheline (2′-di*iso*propylaminoethyl xanthene-9-carboxylate methobromide) is the isopropyl analogue of methantheline and differs from it only in degree of activity (Hambourger, 1952), its actions being qualitatively similar.

Although methantheline and propantheline seem to have considerable atropine-like activity, there have been few comparative studies with atropine in animals. It is the purpose of the present work to provide such a study. At the same time a comparison of the autonomic ganglion blocking activities, and of the curare-like activities, of the two drugs has been made. The local anaesthetic activities have also been investigated.

METHODS

The substances used were methantheline bromide, propantheline bromide, hexamethonium bromide, atropine sulphate and acetylcholine chloride. Doses and concentrations, and comparisons of activity, are given throughout this paper in terms of these salts.

Comparisons of Peripheral Parasympatholytic Activity

Isolated Guinea-pig Ileum.—Steady sub-maximal contractions to ACh were obtained at two-minute intervals. The dose of antagonist was given one minute before the next dose of ACh. The antagonistic effects of three separate doses of atropine, followed by three of methantheline, were measured by the percentage reduction in height of the subsequent contraction due to ACh.

Five such comparative assays of methantheline and atropine and five of propantheline and atropine were performed on pieces of gut from 10 guinea-pigs.

The percentage reduction in height of contraction was plotted against the logarithm of the dose of antagonist; the potency of methantheline or propantheline, compared with atropine, was obtained as the mean of the ratios of doses required to produce 50% inhibition of the height of contraction.

Blood Pressure in the Cat.—In each experiment a suitable dose of ACh was discovered (0.1 to 1.0 μg.) which would give an adequate fall in blood pressure. Comparisons of activity were made by relating the dose of each antagonist needed to inhibit to an equal degree the response to ACh.

Mydriatic Activity in the Mouse.—The method of Ing, Dawes and Wajda (1945) was used.

Intraperitoneal injections of atropine (2, 4, and 8 μg. in 0.2 ml. 0.9% NaCl) were made into three groups of five mice and the pupil diameter measured 15 min. later by viewing the eye under a strong light with the aid of a binocular microscope provided with a scale in the eyepiece. This procedure was repeated twice on the same day in six further groups of five mice, so that 45 animals in all were used. Three doses of methantheline (10, 20, and 40 μg., or 5, 10, and 20 μg.) were investigated in another 45 mice on the same day; four such complete experiments were performed. Similarly three complete experiments with

4, 8, and 16 μg. doses of propantheline, and 2, 4, and 8 μg. doses of atropine, were performed.

The results from the four experiments on methantheline and the three on propantheline were pooled separately and subjected to a suitable form of statistical analysis for calculating the regression line of the dose of mydriatic on mean pupil diameter (Emmens, 1948). From the two regression lines for methantheline and atropine, and the two for propantheline and atropine, the comparisons of potency were made.

Information about the duration of mydriatic activity was obtained by plotting curves relating mean pupil size to the time after intraperitoneal injection of the equiactive doses of 40 μg., 16 μg., and 8 μg. of methantheline, propantheline, and atropine respectively. Groups of 15 mice received atropine and methantheline in one experiment, and atropine and propantheline in another.

Salivary Secretion in the Cat.—The method used was that of Bülbring and Dawes (1945). A steady flow of saliva was produced by an intravenous infusion at a uniform rate (0.5 ml./min.) of 0.004% (w/v) of carbachol in Ringer–Locke solution. Adrenaline hydrochloride (0.002%) was included to offset to some extent the fall in blood pressure produced by the carbachol. The rate of secretion was recorded by a drop timer.

The relative activity of methantheline or propantheline and atropine was found by recording two effects of a dose of one substance between two effects of a dose of the other. This procedure was repeated until an exact match was obtained, or until two doses of one drug were found more and less effective respectively than a fixed dose of the other. The activity was judged by the maximum effect of each injection. Five cats were used for each assay. In each of the ten cats a comparison was made at several dose levels. In eight of the cats the activities of methantheline and atropine or propantheline and atropine in inhibiting the carbachol-induced fall in blood pressure were recorded.

Comparisons of Ganglion Blocking Activity

Nictitating Membrane of the Cat.—The autonomic ganglion blocking property of methantheline bromide was compared with that of hexamethonium in four cats anaesthetized with pentobarbitone sodium, and that of propantheline with hexamethonium in a further four cats, by relating the ability of the drugs to reduce the recorded height of contraction of the nictitating membrane in response to pre-ganglionic nerve stimulation. Stimulation was by square wave shocks, of 800 μsec. duration and 45 c/sec., automatically applied, every 2 min. for 3 to 10 sec., to the central end of the cut vago-sympathetic trunk. Steady contractions were obtained and the drug was given by vein at a fixed time before the subsequent contraction. The reduction in height of the contraction following the drug was expressed as a percentage of the previous contraction height and was plotted against the logarithm of the dose/kg. for each drug. Because the lines were not parallel, the relative potency was obtained by comparing the two log. dose-response lines for each pair of drugs at various response levels.

Blood Pressure in the Cat.—The relative ability of methantheline and hexamethonium to lower the blood pressure was observed in four cats, and that of propantheline and hexamethonium in three. All the cats were anaesthetized with pentobarbitone sodium.

Acetylcholine Hypertension in the Atropinized Cat.—In four cats anaesthetized with pentobarbitone sodium a comparison was made of the relative powers of hexamethonium, methantheline, and propantheline to inhibit the rise in blood pressure caused by the ganglion stimulation produced by large doses of ACh (0.1 to 0.2 mg./kg.) after large doses of atropine. Atropine (1 mg./kg.) was given initially by vein. Several doses of methantheline or propantheline and of hexamethonium were tried in order to find one of each which would almost prevent the rise of blood pressure following ACh.

Comparisons of Curare-like Activity

Cat Sciatic Nerve-Gastrocnemius Preparation.—The cats were anaesthetized with pentobarbitone sodium. Drug injections were given into the proximal side of the ligatured ipsilateral external iliac artery. The sciatic nerve was stimulated by single supra-maximal thyratron shocks at 10 sec. intervals. In two cats the relative potency of propantheline and methantheline was compared in producing inhibition of the contraction of the gastrocnemius muscle.

Comparisons of Local Anaesthetic Activity

An attempt was made to compare any local anaesthetic activity of methantheline with that of cocaine hydrochloride by the guinea-pig method of Chance and Lobstein (1944). The local anaesthetic was applied to both eyes and the corneal reflex tested 45 sec. later and at every minute for 5 min. The number of times the reflex was present out of a possible maximum of 10 for both eyes was recorded for several concentrations of methantheline (5, 2.5 and 1%) and cocaine hydrochloride (0.2%). The substances were dissolved in 0.9% NaCl.

Information regarding the duration, intensity, and any evidence of irritant properties of methantheline and propantheline as local anaesthetics was obtained by limited experimentation in two human subjects. Injections of 0.2 ml. of solutions of methantheline, propantheline or procaine hydrochloride were made into the skin of the flexor surface of the forearm. The solutions were made up in 0.9% NaCl; control injections were of saline alone.

RESULTS

Peripheral Parasympatholytic Activity

Guinea-pig Ileum.—The results of assays on guinea-pig ileum show propantheline to have a mean potency (relative to atropine=1) of 1.95±

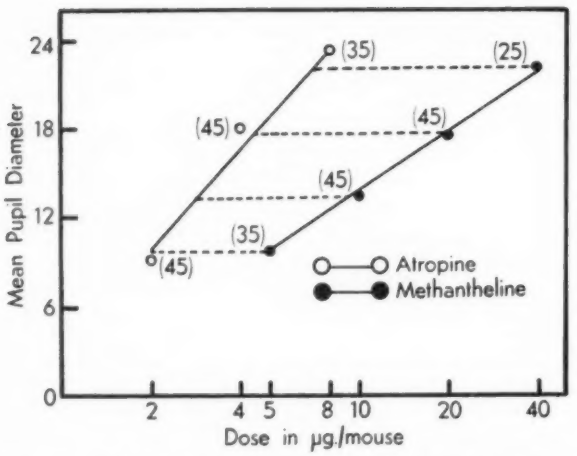

FIG. 1.—The log dose-response lines for methantheline bromide and atropine sulphate in producing mydriasis in the mouse. Ordinate: mean pupil diameter in arbitrary units of the microscope eyepiece scale. Abscissa: µg. per mouse intraperitoneally. The number of animals used for each point is indicated on the graph. The points shown on the graph are the observed mean values. The dose-response lines are calculated. The horizontal interrupted lines indicate the response levels at which the potency comparisons were made.

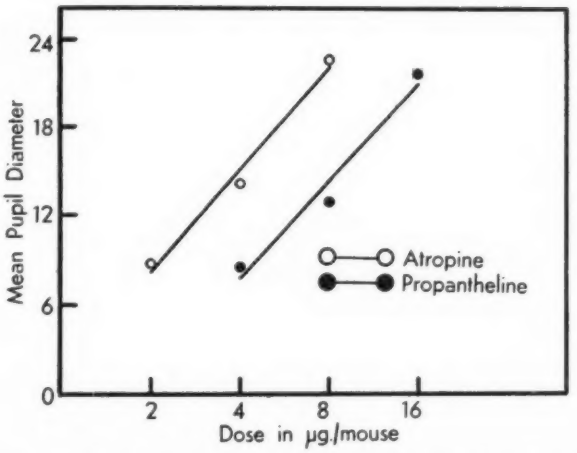

FIG. 2.—The log dose-response lines for propantheline bromide and atropine sulphate in producing mydriasis in the mouse (45 mice for each dose). Ordinate: mean pupil diameter in arbitrary units of the microscope eyepiece scale. Abscissa: µg. per mouse intraperitoneally. Points on graph are observed mean values. Dose-response lines are calculated.

0.08 S.E. (5 expts.) compared with 1.73±0.19 S.E. (5 expts.) for methantheline.

Intravenous Acetylcholine in the Cat.—In four cats the ratio of activity of methantheline to atropine lay between 0.7 and 1.0; in a further four cats the drugs were about equiactive.

Mydriasis in the Mouse.—The two log dose-response lines for methantheline and atropine are shown in Fig. 1 and those of propantheline and atropine in Fig. 2. The points shown on the graph are the observed mean values, whereas the dose-response lines are calculated.

As can be seen, the log dose-response lines for methantheline and atropine are far from parallel; it is therefore impossible to make an accurate estimate of the relative potency of the two drugs. An average has been taken of the relative potencies calculated at four response levels, and these levels are represented by the horizontal dotted lines between the regression lines. The regression lines for propantheline and atropine, however, are parallel. The mean relative potency of methantheline and atropine (=1) is 0.28 and the relative potency of propantheline and atropine (=1) is 0.47.

The durations of action of methantheline and atropine are compared in Fig. 3 and of methantheline and propantheline in Fig. 4. Doses of drugs used were those known to be equiactive at 15 min. The time for half recovery to the pre-injection diameter for methantheline is much less than for atropine (45 min. and 120 min. for 40 µg. and 8 µg. respectively of methantheline and atropine). Propantheline, however, approached atropine in time for half recovery (67.5 min. and 77.5 min. for 16 µg. and 8 µg. respectively of propantheline and atropine).

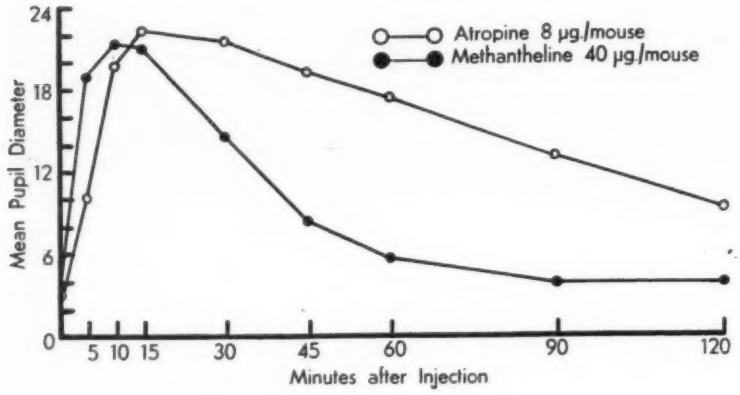

FIG. 3.—Duration of mydriasis in the mouse produced by 40 µg. of methantheline bromide is much less than that following 8 µg. of atropine sulphate intraperitoneally. These doses were equiactive at 15 min. Ordinate: mean pupil diameter from 15 mice in arbitrary units of the microscope eyepiece scale. Abscissa: time after injection in min.

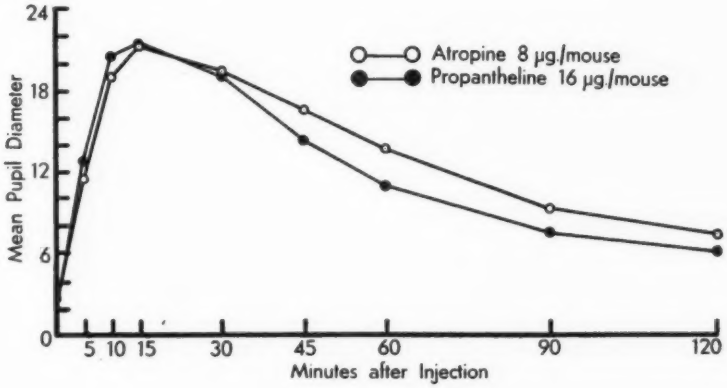

FIG. 4.—Duration of mydriasis in the mouse produced by 16 µg. of propantheline bromide is similar to that following 8 µg. of atropine sulphate intraperitoneally. These doses were equiactive at 15 min. Ordinate: mean pupil diameter from 15 mice in arbitrary units of the microscope eyepiece scale. Abscissa: time after injection in min.

Salivary Secretion.—The results of several comparative assays of methantheline and atropine from each of five cats and those of several assays of propantheline and atropine in each of a further five cats were averaged. The mean relative potency of methantheline (relative to atropine=1) calculated from all the results is 2.23±0.37 S.E. (5 expts.) and that for propantheline 2.95±0.41 S.E. (5 expts.).

In inhibiting the hypotension produced by the infusion of carbachol, methantheline and atropine were approximately equipotent, whereas propantheline had a mean potency of 2.7 (relative to atropine=1).

Ganglion Blocking Activity

Nictitating Membrane of the Cat.—The extent of the fall in blood pressure produced by hexamethonium was such that a dose which would cause over 50% inhibition of contraction could not be given in some cases without fear of killing the animal. The log dose/kg.-response lines for the action of methantheline and propantheline and hexamethonium on the nictitating membrane are not parallel; estimates of relative potency were therefore made at several response levels and the mean calculated for each experiment. Methantheline has a mean potency (relative to hexamethonium=1) in these experiments of 0.40±0.06 S.E. (4 expts.) and propantheline (relative to hexamethonium=1) of 0.63±0.08 S.E. (4 expts.) (Fig. 5).

In two cats it was found that 5 mg./kg. of methantheline or of propantheline had no effect on the contraction of the nictitating membrane produced by intravenous adrenaline.

Reduction in Blood Pressure.—In three cats, 0.5 mg. hexamethonium produced a fall in blood pressure equal to that due to 5 mg. of methantheline and in three other cats 5 mg. of propantheline produced a fall in blood pressure equivalent to from 2.5 to 4 mg. of hexamethonium.

ACh Hypertension in the Atropinized Cat.—Hexamethonium and methantheline were equiactive: propantheline was twice as active as hexamethonium.

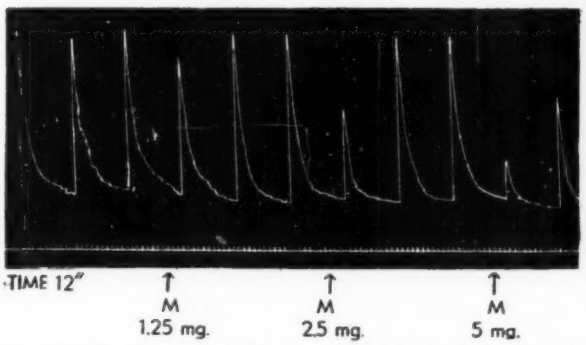

FIG. 5.—A typical record showing the effect of methantheline bromide (M) in inhibiting the contraction of the nictitating membrane of the cat in response to pre-ganglionic sympathetic stimulation. Cat anaesthetized with pentobarbitone sodium. Time intervals, 12 sec.

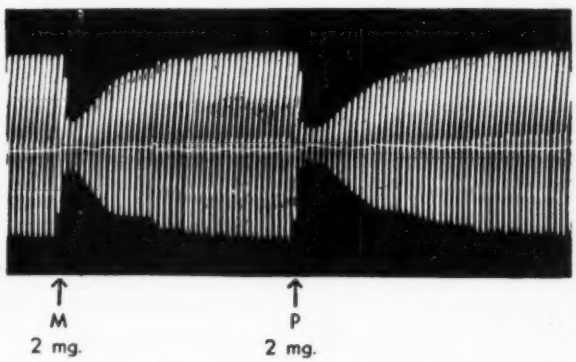

FIG. 6.—Record of the contractions of gastrocnemius muscle of the cat in response to stimulation of the sciatic nerve by single supramaximal shocks from a thyratron stimulator at a rate of 5/min. The inhibitory effects of 2 mg. of propantheline bromide (P) and 2 mg. of methantheline bromide (M) given intra-arterially are seen to be equal. Cat anaesthetized with pentobarbitone sodium.

Curare-like Activity

Propantheline and methantheline showed curare-like activity only in very large doses. They were approximately equiactive. The effects of 2 mg. of propantheline and of methantheline are shown in Fig. 6.

Local Anaesthesia

Using three concentrations of methantheline (5%, 2.5%, 1% in saline) instilled into the guinea-pig eye, no local anaesthetic activity could be demonstrated in any of nine guinea-pigs. Compared with 0.2% cocaine hydrochloride, it produced marked signs of irritation, especially with the 5% and 2.5% concentrations. Similar results were obtained with propantheline in six guinea-pigs, except that there was some evidence of anaesthesia with a 5% concentration. As with methantheline, marked signs of irritation were produced by the 2.5% and 5% solutions.

Experiments in man confirmed the irritant action shown in the guinea-pigs. Intracutaneous injections were followed by the development of a spreading weal and surrounding flare of vasodilatation. Methantheline 1% in two subjects produced anaesthesia of slow onset but prolonged duration in the skin overlying the weal produced by the injection. Several minutes were needed for the full development of anaesthesia, but it lasted for 6 to 7 hr. This is in contrast to the immediate anaesthesia produced by 0.25% procaine, and which continues for only 25 to 30 min. A higher concentration of methantheline, 2%, produced anaesthesia of slightly more rapid onset and of much longer duration. There was still some degree of anaesthesia 24 hr. later. The low concentration of 0.5% methantheline had a similar effect to the 1% concentration. After 1 and 2% methantheline, scarring and hypersensitivity of the skin were present for at least two weeks. Exactly similar results were obtained with propantheline, using 5%, 2.5%, and 1% solutions. It is interesting to note that 0.1 c.c. of each of these solutions intradermally (equal to approximately 8 mg.) in each subject produced complete arrest of salivary secretion and an increased pulse rate. The effect on salivary secretion lasted for approximately five hours. Inhibition of gastro-intestinal activity was suggested by a feeling of fullness in the stomach, and loss of appetite.

Discussion

The present results with methantheline differ considerably from those of other workers—notably Lehman and Knoeffel (1944). As far as can be ascertained no systematic pharmacological examination of the properties of propantheline has yet been reported, apart from that of Hambourger (1952).

Lehman and Knoeffel (1944) found methantheline to be 100 times weaker than atropine in inhibiting salivary secretion. Although their assay was done on cats, their method was totally different from that used here. They used pilocarpine as the sialogogue, and both antagonist and sialogogue were injected subcutaneously, in contrast to the intravenous route used by Bülbring *et al.*, and by which we have found methantheline to be twice as effective as atropine.

Lehman and Knoeffel's results for the relative potencies of methantheline and atropine in preventing the vasodepressor action of ACh also differ from ours. They found methantheline less potent than atropine in this respect, whereas we found the two drugs to be almost equipotent. This difference is difficult to explain, but may depend on the fact that dogs were used in their experiments and cats in ours.

From the present work one can say that propantheline is slightly more active than methantheline, but this increase in activity is roughly equally distributed between its peripheral and autonomic ganglion blocking activity. Hambourger (1952) found propantheline approximately five times more powerful than methantheline against ACh-induced spasm of guinea-pig gut, and approximately twice as active as methantheline in producing paralysis of the superior cervical ganglion. We found no very significant difference between the antimuscarinic activity of propantheline and methantheline, though our results in the nictitating membrane experiments do correlate well with Hambourger's. No satisfactory explanation can be offered for the former anomaly.

The local anaesthetic property of methantheline on the rabbit eye, shown by Lehman and Knoeffel (1944), could not be demonstrated on the guinea-pig eye, though human experiments indicate that both propantheline and methantheline have some local anaesthetic activity when given intracutaneously. How specific this is is not very certain. It may be that methantheline and propantheline damage the nerve endings in the skin, for the injected area remains scarred for several weeks. The skin does not completely regain its normal sensitivity for some time, anaesthesia being followed, for several days after the injection, by a period of hypersensitivity of the protopathic type.

The curare-like activity of both compounds is equal, but very weak (Fig. 6), 2 mg. of each drug

intra-arterially being needed to produce a moderate effect.

One main difference between the two drugs appears to be their duration of action. In the mouse mydriasis experiments the duration of action of propantheline closely approximated that of atropine, whereas the action of methantheline was much shorter.

Although propantheline and, to a lesser extent, methantheline, approaches hexamethonium in ganglion blocking activity, this activity is only present in a dose range, or concentration range, greatly in excess of that necessary for parasympathetic blockade. It seems, therefore, that ganglion blockade is not likely to be responsible for any therapeutic effects when these substances are used clinically—unless a dose range is used which would produce pronounced signs of peripheral parasympathetic blockade.

Clinical reports have stressed the advantages of methantheline and propantheline as atropine substitutes—in respect of relative absence of side-effects—in doses adequate for treating certain gastro-intestinal disorders. Propantheline is claimed to be better than methantheline from this point of view. It may be argued that the relative infrequency of serious side-effects with these drugs is further evidence that their ganglionic blocking activity contributes little if anything to their effectiveness in clinical use.

Summary

1. Propantheline and methantheline, two atropine-like agents claimed to have in addition considerable ganglion blocking activity, have been compared with atropine and hexamethonium in their power to produce peripheral parasympathetic blockade and autonomic ganglion blockade respectively.

2. Both are more potent antimuscarinic agents than atropine, propantheline having 1½ to 2 times the activity of methantheline.

3. Their ganglion blocking potency, which is exhibited in a much higher dosage range, varies from 1/10 to twice that of hexamethonium, depending on the test preparation used. Propantheline is, again, the more active.

4. Both drugs have some weak curare-like action. They possess little, if any, local anaesthetic activity.

5. The significance of these findings, in relation to previous work and to the clinical effects of the drugs, is discussed.

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Brit. J. Pharmacol. (1954), **9**, 224.

THE BLOOD ANTICOAGULANT EFFECT OF SHORT CHAIN-LENGTH DEXTRAN SULPHATES

BY

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Several previous papers have shown that dextran sulphate of high molecular weight has toxic properties, but that of molecular weight about 7,000, corresponding to a chain-length of 20 glucose units, is sufficiently non-toxic for clinical use. The relation between the degree of sulphation and anticoagulant activity for molecules of this size has been explored (Ricketts, 1952a). In extending the work to compounds of shorter chain-length, some experiments were made with sulphuric esters of oligosaccharides in the maltose series, because they were the only oligosaccharides characterized at the time. Since then the separation and identification of dextran oligosaccharides, by chromatography of their benzyl glycosylamine derivatives (Bayly and Bourne, 1952), have enabled exploration to be continued.

Sulphuric esters of the lower members of the maltosaccharide series showed less anticoagulant effect in rabbits than was expected on the basis of activity measurements *in vitro* (Ricketts, 1952b). This paper describes the preparation of some dextran sulphates of short chain-length, their anticoagulant activity *in vitro*, and their effect upon the blood clotting time of the rabbit.

MATERIALS AND METHODS

Two series of compounds, U and O, were made. The U series was obtained by hydrolysing dextran to varying extents and sulphating the product; these compounds were extremely polydisperse in molecular composition. Saccharides for the O series were prepared from a single hydrolysate of dextran by chromatographic separation on a charcoal column. While it was not possible to isolate individual saccharides in sufficient amounts, mixtures of several components—identified by paper chromatography of their benzyl glycosylamines, and by determination of the mean chain-length from the reducing power of the single terminal aldehyde groups—were obtained. These saccharides were then sulphated. The detailed procedures were as follows:

Preparation of Short Chain-length Polydisperse Dextran Sulphates (U Series)

Hydrolysis.—Dextran (60 g.) of the type used as a plasma substitute (intrinsic viscosity 0.33) was partially hydrolysed by heating a thoroughly stirred solution 5.45% w/v in 0.82 N-sulphuric acid, on a boiling water bath for the times shown in Table I. These times were selected, on the basis of a preliminary experiment, to yield dextrans of mean chain-length 20, 10, 7, and 5 glucose units. After cooling to stop the hydrolysis the solution was dialysed and concentrated to 100 ml. Dextran was precipitated by addition of 500 ml. ethanol, ground to a powder in ethanol, washed with ether, and dried over phosphorus pentoxide *in vacuo*. Yields are quoted in Table I as a percentage weight of the initial dextran. They decrease as the chain-length decreases on account of the proportionately greater conversion to glucose.

Sulphation.—Ten grams of each of these dextrans were sulphated, using 15 ml. chlorosulphonic acid in 70 ml. pyridine, and the dextran sulphate was isolated as described in a previous paper (Ricketts, 1952a). The weight of dextran sulphate obtained and its sulphur content are recorded in Table I. Theoretical yield was calculated on the assumption that all the dextran could have been converted to dextran sulphate of the observed sulphur content. The percentage of the theoretical yield actually isolated is shown. Intrinsic viscosities were determined.

TABLE I

PREPARATION AND PROPERTIES OF POLYDISPERSE SHORT CHAIN DEXTRAN SULPHATES (U SERIES), SHOWING DECREASING ANTICOAGULANT ACTIVITY *IN VITRO* WITH DECREASING INTRINSIC VISCOSITY

Serial No.	Dextran		Dextran Sulphate			
	Hydrolysis Time (min.)	Yield %	Yield %	% S in Na Salt	Intrinsic Viscosity	Anticoagulant Activity *in vitro* (u./mg.)*
U 20	17	80	67	18·8	0·049	19·7
U 10	36	53	69	17·8	0·038	9·4
U 7	51	23	52	17·9	0·028	8·1
U 5	72	12	49	18·2	0·024	5·1

* Assayed against a dextran sulphate standard with a heparin activity of 15 u./mg.

Preparation of Short Chain-length Slightly Disperse Dextran Sulphates (O Series)

Hydrolysis.—Dextran (30 g.) of the type used as a plasma substitute, intrinsic viscosity 0.33, but prepared from the B 512 strain of *Leuconostoc mesenteroides* to

minimize the branching in the product, was partially hydrolysed by heating a thoroughly stirred solution, 5.45% w/v in N-sulphuric acid, on a boiling water bath for 90 min. This time was calculated on the basis of a preliminary experiment to yield a product having one quarter of the reducing power of glucose, i.e., of mean chain-length 4 glucose units. After cooling to stop the hydrolysis the solution was neutralized with sodium hydroxide and brought to a final volume of 500 ml.

Chromatography.—The methods of Whistler and Durso (1950) and Bailey, Whelan, and Peat (1950) were modified slightly. A column 60 cm. long by 4.5 cm. diameter was packed for a length of 30 cm. with a mixture of equal weights of Hyflo Super-Cel (Johns Manville Co.) and washed activated charcoal (B.D.H.). The column was wet with water, and the dextran hydrolysate (500 ml., optical rotation +17.41° in 20 cm. tube) was run slowly on to the adsorbent. The adsorption and subsequent elution were followed by optical rotation measurements in a 20 cm. polarimeter tube. The column was eluted successively with water, 5, 15, 25, and 35% v/v ethanol. Fig. 1 summarizes these operations. Appropriate portions of the eluate were combined as illustrated to form five fractions, A to E.

Solvents were removed by distillation under reduced pressure and the saccharides were recovered by precipitation from syrupy solution with excess ethanol. Fraction A, 0.04 g., was mainly glucose with a trace of sodium sulphate and was discarded. The other fractions, B, 1.1 g.; C, 3.1 g.; D, 6.0 g.; and E, 0.67 g., were sulphate free.

Characterization of Fractions.—Reducing power was determined (Schaffer and Hartman, 1921) on materials dried to constant weight *in vacuo* at 60° C. This provided the estimate of mean chain-length recorded in Table II. The individual saccharides present in each fraction were revealed by chromatography of their benzyl glycosylamines using the method developed by Bayly and Bourne (1952). Fig. 2 shows one of the chromatograms obtained. This chromatogram includes as a reference spot pure 1:6 penta-saccharide. The penta-saccharide mixed with a little glucose was applied to the paper at R, the other fractions being applied as indicated. The solvent was butanol 40, ethanol 12, water 20, 0.88 ammonia 1, parts by volume. From the chromatogram it may be inferred that fraction B contains tetra- and penta-saccharides, fraction C penta-, hexa-, and probably hepta-saccharides, fraction D hepta- and higher saccharides. Fraction E is composed of saccharides too large to separate by chromatography.

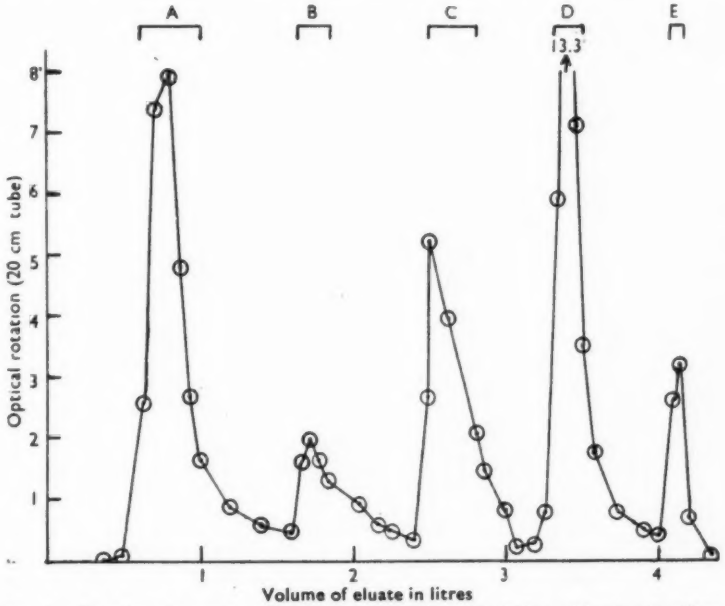

FIG. 1.—Showing changes in optical rotation during the elution of dextran fractions from a charcoal column. Portions of the eluate were combined as indicated to provide fractions A to E.

TABLE II

PREPARATION AND PROPERTIES OF SLIGHTLY DISPERSE SHORT CHAIN DEXTRAN SULPHATES (O SERIES), SHOWING RELATION OF ANTICOAGULANT ACTIVITY *IN VITRO* WITH MEAN CHAIN-LENGTH

Serial No.	Dextran		Dextran Sulphate			
	Weight g.	Mean Chain-Length	Yield of Ester %	% S in Na Salt	Intrinsic Viscosity	Anticoagulant Activity *in vitro* (u./mg.)*
OE	0·67	17·8	54	18·2	0·038	14·3
OD	5·85	9·4	78	20·0	0.029	8·6
OC	3·1	5·7	75	18·8	0·020	8·5
OB	1·1	4·4	75	18·7	0·018	8·5

* Assayed against a dextran sulphate standard with a heparin activity of 15 u./mg.

Sulphation.—Some 0.8 to 5 g., according to the amount available, of each of these fractions was sulphated, using 2 ml. chlorosulphonic acid per gram of dextran and 20 to 50 ml. of pyridine. The yield, sulphur content, and intrinsic viscosity of each preparation is shown in Table II.

The Stability of the Dextran Sulphate Preparations

It was found that the U series preparations slowly decomposed, the powder form in the course of several months and solutions in several weeks. Preparations U 5 and U 7 decomposed first, followed by U 10; U 20 appears to be indefinitely stable. On the other hand the O series compounds all appeared to be indefinitely stable. This was attributed to the presence of glucose sulphate in preparations of the U series and its absence from preparations of the O series. Experimentally, the mixture obtained by sulphating glucose and isolating the glucose sulphates as sodium salts decomposed in a period of months. Decomposition always takes the form of increasing acidity, the production of reducing substances and, ultimately,

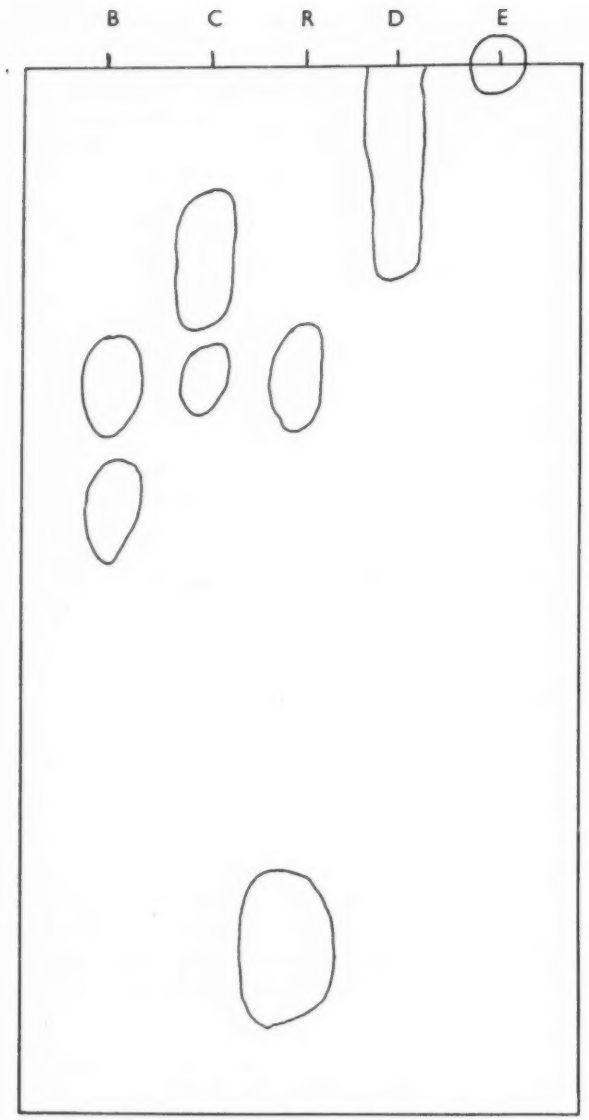

FIG. 2.—Chromatogram showing individual saccharides in the dextran fractions of Table II. The reference spot at R was a mixture of glucose with pure penta-saccharide.

charring of powders. It seems likely that decomposition is initiated by traces of acid and subsequently becomes autocatalytic.

Anticoagulant Activity

Anticoagulant activity *in vivo* was observed after injection of the sodium salt of the dextran sulphate dissolved in 0.5 or 1 ml. saline into the marginal ear-vein of a rabbit. Clotting time was measured by the method of Dale and Laidlaw (1911) on blood taken from the opposite ear, the first drop of blood being discarded to minimize contamination with tissue thromboplastin.

Anticoagulant activity *in vitro* was measured using recalcified horse plasma (Ricketts and Walton, 1953). To avoid the uncertainties of comparing various dextran sulphates with heparin a dextran sulphate preparation was adopted as a standard. This standard had previously been compared with heparin in several types of assay and its activity found to be about 15 u./mg. This figure was used in calculating the activities of the different preparations.

RESULTS

The effect of the polydisperse (U series) preparations upon the blood clotting time of a rabbit is shown in Fig. 3. Their efficacy, on a weight basis, in raising the clotting time diminishes with decreasing molecular size. This is well illustrated by the effect of 120 mg./kg. of the short chain preparation U 7 being less than the effect of 15 mg./kg. of the preparation U 20, which has a longer molecular chain. Another useful comparison is the result obtained with the dextran sulphate fraction I 7.6 which has the biological properties advocated for a clinical anticoagulant (Ricketts and Walton, 1953). The effect with this fraction at 10 mg./kg. is greater than the effect of U 10 at 15 mg./kg.

The effect of the less disperse preparations (O series) upon the blood clotting time of a rabbit is shown in Fig. 4. Considering the effects at a dose of 10 mg./kg. it is clear that there is a decreasing effect on clotting time with decreasing molecular weight. This is confirmed by the result with the long chain preparation OE, which, in a dose of 6 mg./kg., has a greater effect than 10 mg./kg. of the shorter chain preparations OC and OB.

The *in vitro* anticoagulant activities of members of the U and the O series of preparations are given in the last column of Tables I and II.

DISCUSSION

Dextran sulphate shows anticoagulant activity over a very wide range of molecular weight. Large molecules have the undesirable effects of precipitating proteins and aggregating blood platelets. Dextran sulphate with molecules composed of about 20 glucose units has given satisfactory results as a clinical anticoagulant (Ricketts, Walton, van Leuven, Birbeck, Brown, Kennedy, and Burt, 1953). The present study has explored the anticoagulant action of molecules with fewer than 20 glucose units.

Preparations in both the U series and the O series have successively lower molecular weights; the main difference is that the preparations in the U series are considerably more polydisperse than those in the O series.

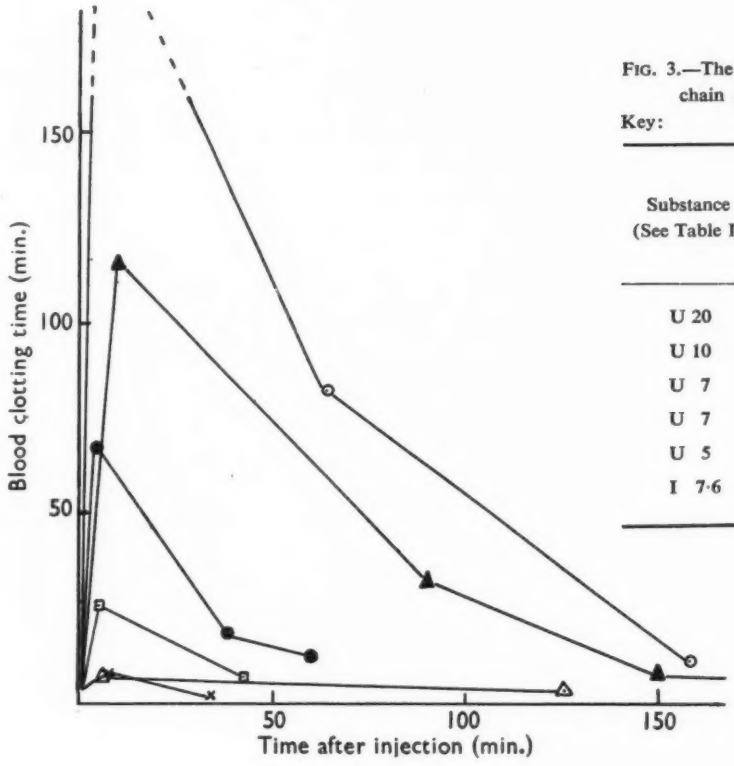

FIG. 3.—The elevation of blood clotting time in a rabbit by short chain dextran sulphate preparations of the U series.

Key:

Substance (See Table I)	Intravenous Dose		Symbol
	mg./kg.	u./kg.*	
U 20	15	270	⊙——⊙
U 10	15	130	⊡——⊡
U 7	15	112	◬——◬
U 7	120	900	▲——▲
U 5	15	70	×——×
I 7·6	10	120	●——●

* As determined by *in vitro* assay.

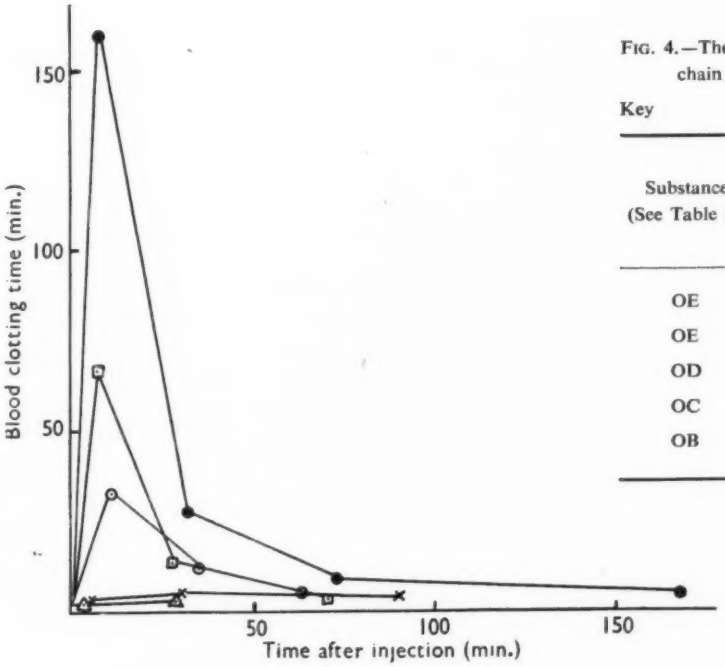

FIG. 4.—The elevation of blood clotting time in a rabbit by short chain dextran sulphate preparations of the O series.

Key

Substance (See Table II)	Intravenous Dose		Symbol
	mg./kg.	u./kg.*	
OE	10	140	●——●
OE	6	80	○——⊙
OD	10	80	⊡——⊡
OC	10	80	◬——◬
OB	10	80	×——×

* As determined by *in vitro* assay.

When anticoagulant activity is measured *in vitro*, the activity of the polydisperse preparations of the U series falls as the average size of the dextran sulphate molecules decreases—as indicated by the intrinsic viscosity (Table I). As might be expected, this fall in activity is not so marked in the O series where the lower members, OC and OB (Table II), do not contain inactive esters such as those of glucose and isomaltose.

The data of Figs. 3 and 4 show that the shorter chain preparations are less effective, on a weight basis, in raising the blood clotting time of rabbits. Thus the fall of anticoagulant activity with chain-length below about 20 glucose units is confirmed.

An alternative comparison of anticoagulant effect *in vitro* and *in vivo* may be made on a potency basis. Comparing the effect of equipotent doses of the O series preparations, shown in Fig. 4, it is clear that the effect on blood clotting time is less with preparations OB and OC, of shorter chain-length, than it is with the longer chain preparations OD and OE. A partial explanation of this may be that whereas potency is measured using plasma the effect *in vivo* is measured on whole blood, so that the inhibition of a different selection of clotting factors is involved. It is probable that these short chain preparations rapidly escape from the blood stream; this would also explain their slight effect upon blood clotting time in the doses used.

Thus molecules of dextran sulphate with fewer than 6 glucose units, though active *in vitro*, do not appreciably contribute to the elevation of blood clotting time *in vivo*.

Summary

1. Investigation of the blood anticoagulant effect of dextran sulphate has been extended into the oligosaccharide region.

2. Anticoagulant activity assayed *in vitro* decreases with decreasing molecular chain-length.

3. The effect upon the blood clotting time of the rabbit decreases more markedly with decreasing size than would be predicted from assay *in vitro*. This may be due to rapid disappearance of the smaller molecules from the blood stream.

It is a pleasure to acknowledge many helpful discussions with Dr. J. P. Bull and Dr. K. W. Walton and to thank Professor J. R. Squire for his encouraging interest in this work.

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METHODS FOR STUDYING THE REFLEX ACTIVITY OF THE FROG'S SPINAL CORD

BY

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This paper outlines a method for testing the actions of drugs on the frog's spinal cord. The preparation described here has proved useful for demonstrating the classical properties of spinal reflexes and is more predictable in its behaviour than the spinal or decerebrate cat. Moreover, the frog preparation is sensitive to many drugs with known actions on the mammalian cord and should be found convenient for testing new compounds. Our primary reason for the development of this method was, in fact, the need for a simple preparation which might help in a search for hypothetical humoral transmitters in spinal reflex arcs (Burgen and MacIntosh, in press; Brock, Coombs, and Eccles, 1952). This interest defined the requirements of our preparation and we consequently sought a system which would provide:

(*a*) isolation of the spinal cord from the systemic circulation in such a manner that spinal reflexes remained excitable for several hours,

(*b*) a means of recording continuously both reflex excitation and reflex inhibition, and

(*c*) a slow flow of Ringer's solution over the active cord.

The method we have found most successful is unusual in that the vascular system is perfused with gaseous oxygen.

METHODS

Principal Method

Perfusion of the Vertebral Canal (Fig. 1).—The Ringer solution used contained 6.5 g. NaCl, 0.14 g. KCl, 0.12 g. $CaCl_2$, 2.0 g. dextrose, and 30 ml. of 0.15 M-sodium phosphate buffer (*p*H 7.4) per litre. This fluid flowed from a reservoir bottle (A), some 30 cm. above the frog, fitted with a Marriotte tube to ensure a constant pressure-head. The outlet from the reservoir supplied the frog through two parallel alternative channels; each channel contained a drop-counter (B, C). The common outlet from the drop-counters was through a T-tap (E), and the flow was adjusted by means of a screw-clamp (G) on the single plastic tube of 1.0 mm. internal diameter leading to the vertebral cannula. The latter was made by grinding off the sharp tip of a No. 18 intravenous needle and sawing off the butt.

During control periods the vertebral canal was supplied through drop-counter B (Fig. 1); test solutions were introduced through the heavy rubber tubing beneath drop-counter C, which was connected to an air-trap (D). The counter used for the test solutions was fitted with a tap (F) for rinsing. The double route for perfusion fluid made it easy to change from the control to a test perfusion and back again without interruption or change of flow.

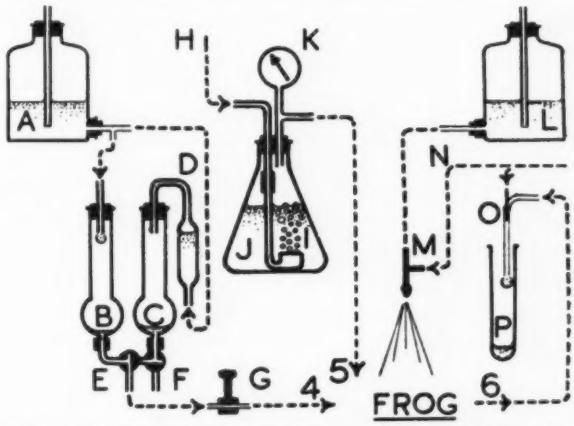

FIG. 1.—A diagram of the apparatus. The numbers 4, 5, and 6 correspond with the same numbers in Fig. 2. A, Reservoir containing Ringer solution fitted with Marriotte tube. B, Drop-counter for control perfusion. C, Drop-counter for test solutions. D, Air trap. E and F, T-taps. G, Screw clamp on plastic tube leading to cannula. H, Gas line from cylinder. I, Bubbler for saturating gas with water vapour. J, Ringer solution in saturating flask. K, Pressure gauge. L, Reservoir containing Ringer solution for spray. M, Atomizer for spraying frog's skin and muscle. N, Compressed air line. O, Hypodermic needle pushed into fine plastic tube. This provides suction. P, Test tube for collecting effluent from the vertebral canal. 4, Ringer fluid supply to vertebral canal. 5, Gas supply to vascular system. 6, Fine plastic tubing for collection of effluent from the vertebral canal.

Perfusion of the Vascular System with Gas (Fig 1).—Gas was supplied from standard commercial cylinders fitted with pressure regulators. It was saturated with water vapour by passing it in small bubbles through water or Ringer solution (I, J) from which it ran through 1.0 mm. bore plastic tubing to a

cannula, made from a No. 20 intravenous needle. A gauge (K) attached to the saturating vessel registered pressure. A mixture of 97% O_2 and 3% CO_2 was used for most of the experiments, but the effects of some other gas mixtures were investigated.

Operative Procedure.—The frogs used were all *Rana pipiens*. After the brain had been destroyed by pithing, the skin of the thigh was slit over the biceps femoris muscle, and the distal two-thirds of the muscle freed from adjacent fascia and the underlying blood vessels and sciatic nerve. A thread was tied around the tendon close to its insertion at the knee. The skin was then slit over the vertebral column and the spinal cord was exposed in the brachial region by dissecting away the tops of the neural arches. Further preparation of the vertebral canal for perfusion was left until after the gas perfusion of the vascular system had been established.

The frog was then turned on to its back and the abdominal cavity was opened. A ligature was tied around the base of each lung and another around the two systemic arches just superior to the origin of the dorsal aorta. These ligatures excluded the lungs, the viscera, and the posterior part of the frog from the general circulation. The heart was then exposed and the cannula attached to the gas supply line was tied into the truncus arteriosus. The cannula was made from a No. 18 intravenous needle. Perfusion with gas was begun, the efferent gas escaping from a slit made in the sinus venosus. The gas was supplied at constant pressure, between 100 and 140 mm. Hg in different experiments.

The test muscle and the peripheral nerves supplying it were isolated from both perfusions. In spite of this isolation, reflex responses remained satisfactory for periods as long as 6–8 hr.

The frog was turned back on to its belly and the cannula for the Ringer perfusion was pushed forwards into the vertebral canal through the gap made by flexing the joint of the urostyle and the vertebral column proper. If this was properly done the cannula remained firmly in place, with no leakage of fluid around its shank. The Ringer perfusion was then started, the fluid flowing out of the canal in the brachial region.

The frog was pinned firmly in position on the cork board (Fig. 2). If the knee joints are pinned so that the two femurs lie in a straight line, distortion of the record due to occasional movements of the frog is reduced. Finally, the exposed section of the spinal cord was crushed with forceps and removed, leaving a cavity from which the cord perfusate was collected by gentle suction (Fig. 1, O, P; Fig. 2, 6).

Throughout the experiment the exposed tissues and the remaining skin of the frog were kept moist by a spray of Ringer solution (Fig. 1) of the same composition as that used for the cord perfusion except that it contained no dextrose. The atomizer (M) was of the ordinary nasal spray type and was supplied with Ringer from a stock bottle (L) and a constant current of air from the compressed air mains (N).

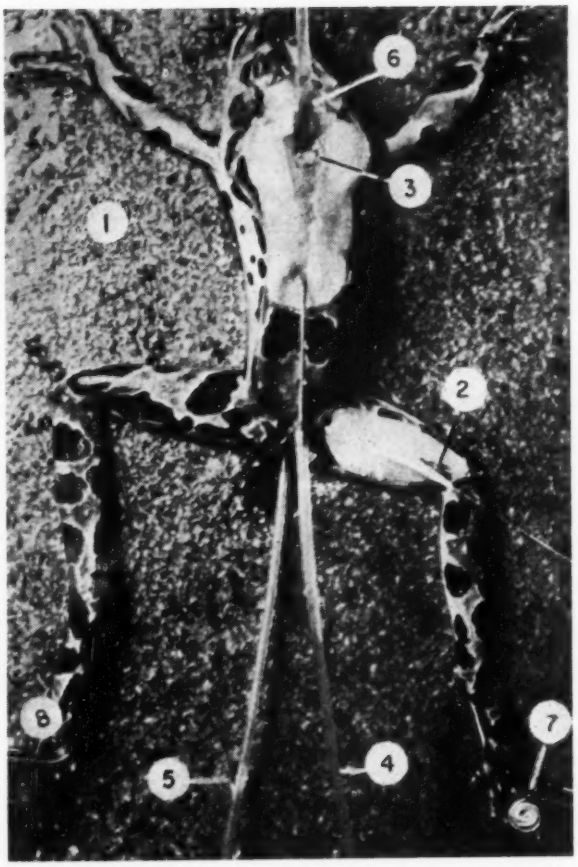

FIG. 2.—A frog prepared for an experiment. 1, Cork mounting board. 2, The biceps femoris muscle. 3, The cut end of the spinal cord. 4, The Ringer solution supply line to the vertebral canal. 5, The gas supply line to the vascular system. 6, The suction tube for collecting the effluent perfusate. 7 and 8, The stimulating electrodes in position on the feet.

Stimulation.—The electrical stimuli applied to the animal's feet were sinusoidal at 60 c.p.s. and variable between 0–20 volts. Two channels of stimulation were available and the strengths of the stimuli applied to each foot of the frog could be varied independently. The timing of the stimulation periods was controlled by standard, commercial, adjustable commutators driven by synchro-motors connected to the A.C. mains (R. W. Cramer & Co.). These were arranged so that :

1. Every n sec. the biceps femoris muscle was reflexly stimulated by a train of stimuli (S_e) of duration d_e applied to the ipsilateral foot. n was variable between 10–120 sec., but was usually 30 sec.; d_e was variable between 0–10 sec., but was usually 1 sec.

2. Every $2n$ sec. a train of stimuli (S_i) of duration d_i could be given to the other foot in order to produce reflex inhibition of the response to S_e. d_i was variable but was set so as to be $2d_e$, and the timing was arranged so that S_i began before S_e and finished after S_e was over.

The bipolar stimulating electrodes were flat coils of silver wire, and these were applied above and below a foot (Figs. 2, 7, and 8).

(In the experiments reported here, stimulation has been regularly applied at two sites only, the foot ipsilateral to the dissected muscle, and the contralateral foot; the responses are flexor responses presumably involving a polysynaptic reflex path. The method should, however, be adaptable to the investigation of monosynaptic reflexes or to other reflexes, evoked by stimuli restricted to the skin or to chosen nerve trunks.)

Recording of the Muscular Contractions.—The tendon of the muscle was severed close to the knee, and the thread already attached to it was passed under a pulley to a lever writing on a slowly moving smoked drum; the muscle lay in its natural position during the experiment. The recorded contractions were isotonic, the muscle working against a weight of 2 g. The electrodes were put in position on the two hind feet and stimulation was carried out at regular intervals, as described above. Since alternate stimuli to the foot on the same side as the dissected muscle were accompanied by stimulation of the contralateral foot, the records show a regular succession of uninhibited and inhibited contractions. Fig. 2 shows the frog prepared for the experiment, and Fig. 3 a control record.

In this record the responses are stable for a period of over half an hour and very regular. It is always possible to produce such a regular record in a good preparation, provided that the cord has been transected behind the brachial enlargement, and that the stimulus strength is such as to produce a maximal or nearly maximal response on ipsilateral stimulation. When the stimulus is well below maximal, the responses often become less regular, as the preparation is easily affected by small changes in the area of contact of the electrodes, or in the afferent input from the skin and viscera supplied by the remaining section of cord posterior to the brachial region. Submaximal stimuli have nevertheless been preferred, since with these it was easier to detect the augmentation of the reflex response which occurred with some procedures. In Fig 4, the submaximal responses in the control period, and again after recovery, were stable; in Figs. 5 and 6 the responses, especially the inhibited responses, show a greater irregularity, but the average level was sufficiently constant for any important change due to altered conditions to be unmistakable. In general, a control period was regarded as satisfactory if the uninhibited responses did not show random variations from the mean of more than $\pm 15\%$ and showed no steady trend.

Other Methods Investigated

Before the adoption of the technique described above, several other methods of maintaining a preparation that would show good reflex activity for some hours were tried. Two of these procedures were successful. Although not suitable for investigating the possible liberation of transmitting substances in the cord, they could be used to test the effects of drugs on spinal reflexes. In neither of the two methods was the vascular system perfused. Circulation was arrested by excision of the heart, and the cord supplied with oxygen and Ringer solution through the vertebral canal perfusion. The first method described below, in which the vascular system was perfused with oxygenated Ringer solution, was a failure.

1. *Double Perfusion using a Ringer Solution in both the Intravertebral and Vascular Perfusion Systems.*—Some attempts were made to supply the cord with oxygenated Ringer flowing through the anterior part of the vascular system, using at the same time a slow intravertebral perfusion with Ringer as a route for the introduction of drugs. No successful preparation was obtained in this way: with low vascular perfusion pressures responses rapidly declined; with high perfusion pressures all tissues became oedematous. The use of gum in the Ringer prevented the oedema, but slowed the perfusion rate so much that even at high pressures oxygenation became inadequate. Attempts to improve this method were abandoned when the gaseous perfusion was found to be effective.

2. *Intravertebral Perfusion with Oxygenated Ringer Solution.*—The vertebral canal was perfused with

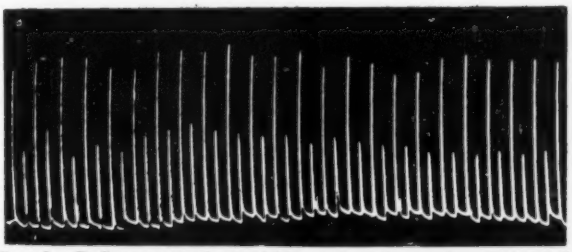

FIG. 3.—Reflex contractions of biceps femoris muscle. A control record. The long strokes are the reflex responses of the muscle to stimulation of the ipsilateral foot, the short strokes the inhibited responses which result when the two feet are stimulated together. Interval between stimuli, 30 sec.

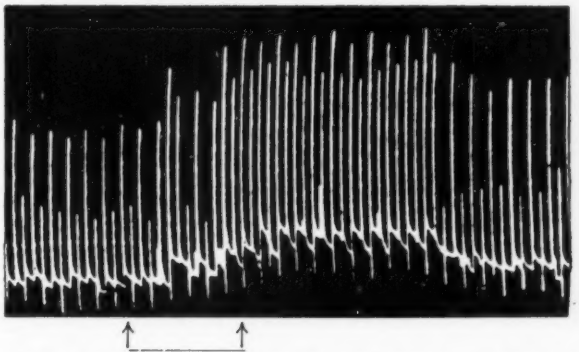

FIG. 4.—Reflex contractions of biceps femoris muscle. The effect of six minutes of anoxia. N_2 was substituted for O_2 during the period between the arrows. Interval between stimuli, 30 sec.

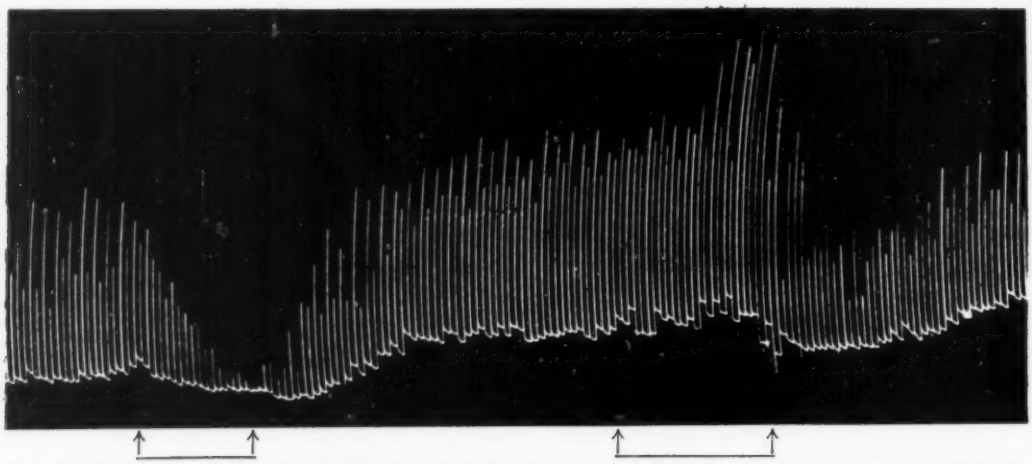

FIG. 5.—Reflex contractions of biceps femoris muscle. The effects of N_2O and anoxia. N_2O was substituted for O_2 for a 10 min. period between the first pair of arrows, N_2 for O_2 during an 11 min. period between the second pair of arrows. The complete recovery from anoxia is not shown; it was a slower process than the recovery from N_2O, which was complete in 7.5 min. Interval between stimuli, 30 sec.

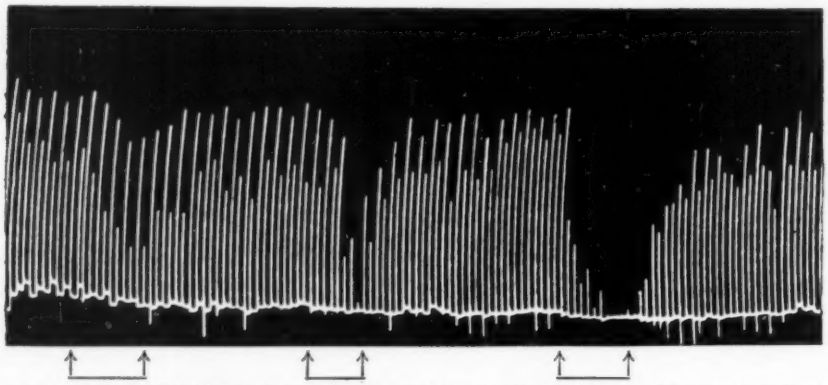

FIG. 6.—Reflex contractions of biceps femoris muscle. The effect of ether. Ether was introduced in solution in the Ringer fluid perfusing the vertebral canal: between the first pair of arrows, ether 10^{-3} for 5 min.; between the second pair of arrows, ether 2×10^{-3} for 2.5 min.; between the third pair of arrows, ether 2×10^{-3} for 4 min. Interval between stimuli, 30 sec. Flow, 0.5 ml./min.

Ringer solution oxygenated by bubbling a mixture of 97% O_2 and 3% CO_2 through the reservoir bottle. This single perfusion will maintain good reflex activity for several hours provided that the flow along the vertebral canal exceeds 3 ml. per min. This method is simple, but the rapid flow not only uses up large volumes of Ringer solution, but would result in such a dilution of any liberated transmitter that the chances of demonstrating its presence would be vanishingly small. If the flow rate is decreased, the reflex responses invariably decline. This decline must be due to insufficient oxygen supply, since a slow flow of Ringer does not depress the responses when oxygen is supplied through the vascular system. In the latter case flow rates as low as 0.05–0.1 ml. per min. have been used successfully.

3. *Intravertebral Perfusion with Ringer and Gas Mixtures.*—In this variant of the single perfusion technique, a hypodermic needle was inserted into the Ringer supply line so that its tip projected into the lumen of the vertebral canal cannula; through it was introduced the O_2 and CO_2 gas mixture. It was possible to adjust the flow of gas so that the jet of bubbles did not jar the cord, which remained reflexly active for a period of hours even with very slow rates of fluid perfusion. This method is more economical of Ringer's fluid than the previous one, and has been used successfully in experiments for testing the effects of drugs and anoxia on the cord. It has the disadvantage that small changes in the rate of flow of gas alter the Ringer perfusion rate, so that it is more difficult to keep experimental conditions constant than with the double perfusion system described earlier.

RESULTS

Preliminary tests of the sensitivity of the preparation to anoxia and a number of drugs have demonstrated its usefulness. The concentrations of drugs given below are not necessarily the lowest concen-

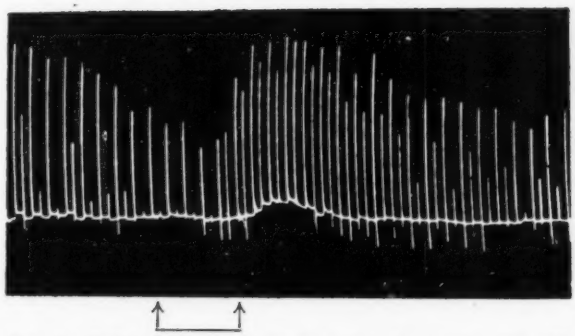

FIG. 7.—Reflex contractions of biceps femoris muscle. The effect of nicotine tartrate. Ringer solution containing nicotine tartrate in a concentration of 2×10^{-5} flowed through the vertebral canal for 6 min. in the period between the arrows. Interval between stimuli, 30 sec. Flow, 0.75 ml./min.

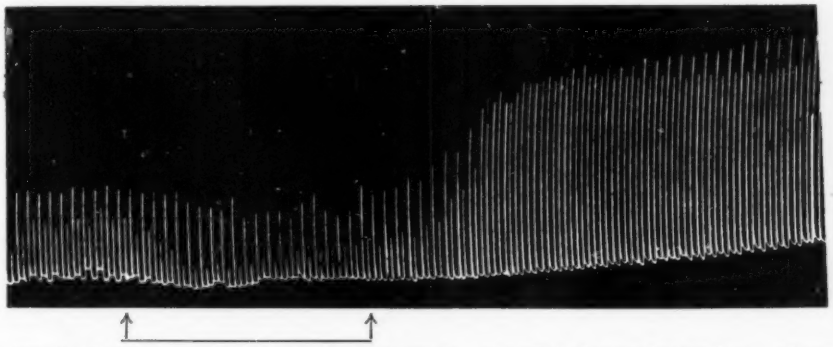

FIG. 8.—Reflex contractions of biceps femoris muscle. The effect of strychnine sulphate. Ringer solution containing strychnine sulphate 10^{-4} was perfused through the vertebral canal for 24 min. during the period between the arrows. Interval between stimuli, 30 sec. Flow, 0.1 ml./min.

trations which could produce an effect, as a wide range of concentrations has not yet been used. Moreover, the effect of any drug upon the cord will depend on the flow of saline and the duration of exposure.

Effects of Changing the Gas Mixture in the Vascular System.—Anoxia was produced by substituting N_2 for O_2, the concentration of CO_2 being kept at 3% in both cases. An increase of the reflex contractions occurred, as it does in mammals during anoxia. Kirstein (1951) has reviewed the pertinent literature and Brooks and Eccles (1947) have shown that the synaptic potentials of mononeurones are increased during anoxia. Our records show, in addition, that stimuli which produced definite inhibition in the control period became ineffective in depressing the reflex response during anoxia. If the period of anoxia was short, these effects were rapidly and completely reversible (Fig. 4). Continued anoxia depresses and finally abolishes both the uninhibited and the inhibited reflex responses.

Nitrous oxide substituted for O_2 had a purely depressant effect, which was rapidly reversible on the readmission of O_2. This immediate depression contrasts with the initial potentiating effect of anoxia. Fig. 5 shows the very different effects of N_2O and N_2 on the same preparation.

Effects of Drugs Introduced into the Ringer Perfusing the Vertebral Canal.—Ether was introduced dissolved in the perfusate of the vertebral canal. Like N_2O, its effect was to depress the reflex contractions, the degree of depression depending on the concentration and the duration of the exposure of the cord to it (Fig. 6).

Nicotine tartrate increased the reflex responses. There was typically a rise in tone with the first dose of nicotine (Fig. 7). Subsequent doses produced less or no rise in tone, and smaller increases in the reflex contraction.

Strychnine sulphate augmented the reflex response, and inhibitory stimuli became ineffective. Under the conditions of these experiments, the effects of strychnine were slow to develop (Fig. 8). It can be seen from the record that inhibition not only failed, but ultimately bilateral stimulation produced a greater reflex contraction than did ipsilateral stimulation alone. The same phenomenon has been reported in the toad by Kato (1934). In mammals a similar increase in flexor contractions occurs after the injection of strychnine (Bernhard, Taverner, and Widen, 1951 ; Bradley and Eccles, 1953). Anoxia in the frog (Figs. 4 and 5) produces comparable increases in the muscular response, with the same failure of inhibitory stimulation.

DISCUSSION

The use of a frog rather than a mammal simplifies both the operative procedure and the subsequent conduct of the experiment: preliminary anaesthesia is not necessary, the cord is more accessible, and muscles isolated from any circulation remain capable of contraction for hours ; in no case has an experiment been terminated because of fatigue of the muscle. An added convenience is that the experiment can be carried out with the animal at room temperature if a sufficient supply of oxygen to the cord can be arranged.

The use of the double perfusion system described here for the frog allows (1) changes in the composition of the gas mixture in the vascular system without interfering in any way with the Ringer

perfusion of the vertebral canal, and (2) changes in the chemical composition of the fluid perfusing the canal without interference with the oxygen supply to the cord. It has the added advantage that very slow flows of Ringer may be used, so that any substances liberated during cord activity will not be too greatly diluted in the perfusate, which is an important consideration if the liberation of synaptic transmitters in the cord is to be demonstrated.

A simple preparation in which the muscle responses are recorded as described, but the natural circulation of the frog is left intact except for the damage inevitable in pithing, has proved very useful in teaching for the demonstration of the classical properties of spinal reflexes. Electrodes may be placed in any suitable position, and the facilitory or inhibitory effects of changing the stimulus site, frequency, or strength studied. If in addition a Ringer perfusion of the vertebral canal is used, the effects of drugs or anaesthetics on the reflex activity of the cord can be readily demonstrated. Such a method does not meet the more rigid requirements for research, since with the natural circulation intact the diffusion in drugs into the spinal vessels would result in their distribution to tissues other than the cord.

Many of the previous experiments in which the effects of varied stimuli and drugs were tested on amphibian spinal reflexes have been done on spinal frogs kept alive on their normal circulation. Whether the drugs were injected intravascularly (Bremer and Moldaver, 1934 ; Bonnet and Bremer, 1937) or dripped on to an exposed section of cord (Lefebvre and Minz, 1936), such experiments do not exclude the possibility that part of the observed change in the responses is due to contributing effects from actions of the drug elsewhere than on the cord itself ; and if the perfusate is to be tested for transmitters, blood is an unsuitable vehicle. Torda (1940) in her experiments perfused a spinal toad with Ringer, isolating her test muscle, the gastrocnemius, from communication with the rest of the body except via the sciatic nerve, thus excluding the muscle from the effect of the circulating drug. This method again, because it does not restrict perfusion to the spinal cord, is unsuitable for our purposes.

A method similar in some respects to ours has recently been published by Häusler and Sterz (1952). The test frog (*Rana esculenta*) was pithed and eviscerated. Perfusion of the intradural space with Ringer containing dextrose was effected by putting in a cannula through the pithing hole, and cutting across the posterior end of the cord to allow the fluid to escape. A second Ringer perfusion supplied the forelimbs, and the flexion of one forelimb to stimulation of the contralateral forelimb was recorded. This preparation gave good reflex responses for hours with flows as slow as 1–2 ml./min. over the cord. The Ringer was saturated at a low temperature with oxygen and allowed to warm to room temperature before it was perfused through the frog (Häusler, personal communication). We have not tried oxygenating Ringer in this way, but our preparations did not remain in good condition on such slow flows of Ringer saturated at room temperature with oxygen. Whether the difference in survival time between our frogs and those of Häusler and Sterz is due to differences in saturation of the Ringer fluid with oxygen, to a lower " room temperature " in Graz than in Montreal, or to a species difference, we do not know, as we have not tried to repeat their experiments.

Kato and his co-workers (Kato, 1934) sometimes used a preparation consisting of the spinal cord with its attached nerve roots, the sciatic nerve, and the gastrocnemius muscle. The effects of drugs on the reflex responses were tested by immersing the cord in the appropriate solution. The reflexes of a frog's completely isolated spinal cord have been studied by Eccles (1946) using the electrical response of a ventral root as the sign of reflex activity. The cord, kept in Ringer solution in a chamber with a stream of oxygen passing through it, gave uniform reflex responses on appropriate stimulation of the dorsal root for a period of hours. The effects of drugs on the reflex could be tested by adding them to the Ringer in the chamber. The method requires that the experiments must be carried out at the low temperature of 10° C. (Eccles, personal communication) if the cord is to survive.

There seems no reason why the method we have described should not be adaptable to the conditions of electrical recording. Some modification would obviously be required if access to the spinal cord or its roots was needed, but the maintenance of reflex activity by oxygen-perfusion should prove useful, since the experiments could then be carried out at room temperature.

Summary

1. Methods of keeping the perfused spinal cord of the frog reflexly active for several hours are described.

2. The usefulness of the different methods in demonstrating the classical properties of spinal

reflexes, and in testing the effects of drugs on uninhibited and inhibited reflex responses, is discussed.

Our thanks are due to the National Research Council of Canada for making this work possible.

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THE USE OF THE RAT'S ISOLATED SEMINAL VESICLE FOR THE ASSAY OF SYMPATHOLYTIC DRUGS*

BY

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The isolated seminal vesicle of the guinea-pig has been used by Brugger (1945), Rothlin and Brugger (1945), and Rothlin (1947) for the evaluation of the sympatholytic effect of the natural and hydrogenated ergot alkaloids; by Stone and Loew (1952) for the assay of sympatholytic (anti-adrenaline) drugs; and by Meier (1950) for the study of antihistaminic drugs. To the best of our knowledge, no information is available on the comparable use of the isolated rat seminal vesicles, although they have been used recently by Haley and Leitch (1954) in an attempt to detect ferritin inhibition of adrenaline responses. The present paper deals with the reaction of the rat's isolated seminal vesicles to adrenaline, acetylcholine, histamine, and a series of anti-adrenaline drugs.

* This paper is based on work performed under contract At-04-1-GEN-12 between the Atomic Energy Commission and the University of California at Los Angeles.

METHODS

The seminal vesicles and associated structures were removed from Wistar strain rats of 250 to 500 g. and placed in Ringer-Locke solution of the following composition: NaCl, 9.0 g.; KCl, 0.42 g.; $CaCl_2,2H_2O$, 0.24 g.; $MgCl_2,6H_2O$, 0.005 g.; $NaHCO_3$, 0.5 g.; anhydrous glucose, 0.5 g.; and distilled water to 1 l.

Greene (1935) reported that the rat's seminal vesicles are contained within the same connective tissue sheath as the coagulation glands. It was found that the latter glands must be carefully removed, without injury to the vesicles, otherwise abnormal responses and reduced sensitivity result. After removal of all extraneous tissue, both vesicles were transected distally to their point of entrance into the ductus deferens.

Both preparations were transferred to the same tissue chamber and attached for dual recording. A 15 ml. tissue chamber, maintained in a water bath at 37.5± 0.2° C., and having a continuous flow of 100 ml. of Ringer-Locke solution per minute, was used in all experiments. Flow was interrupted when drugs were added to the chamber. The bathing solution was continuously oxygenated with a mixture of 95% O_2 and 5% CO_2. All drug dilutions were prepared in 0.9% NaCl and given in a standard volume of 0.5 ml. Preliminary tests showed that ten minutes after the seminal vesicles were placed in the tissue chamber, 30 μg. doses of adrenaline, given at six-minute intervals, produced responses which varied only slightly during the next two hours.

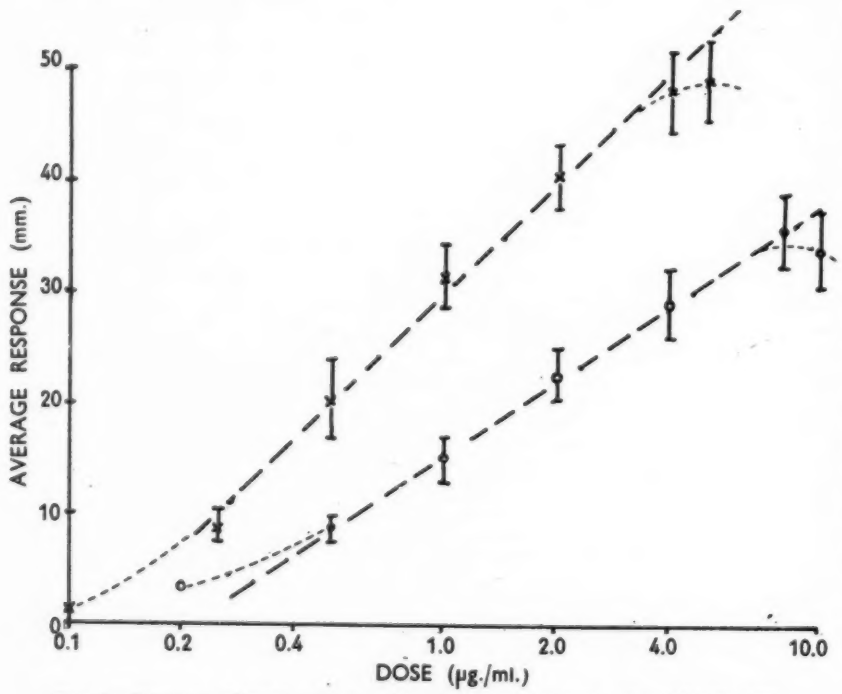

FIG. 1.—Response of rats' seminal vesicles to adrenaline (×) and acetylcholine (O).

In testing sympatholytic drugs, 30 μg. doses of adrenaline were given at six-minute intervals until the last two responses did not vary by more than $\pm 5\%$ from their average. Six minutes later the sympatholytic drug was given and allowed to act for one minute before the standard dose of adrenaline was repeated. Only one dose of blocking drug was given per tissue preparation because of the varying degrees of tissue fixation. However, piperoxane, which can be removed by washing, was alsot ested by giving multiple doses to the same preparation. Three additional doses of adrenaline were given after the blocking drug in order to obtain information on the degree of tissue fixation and the rate of elution of the latter. The results obtained in these experiments were statistically analysed by the Litchfield-Wilcoxon method (1949).

Results

Response to Adrenaline, Acetylcholine, and Histamine.—The response to both adrenaline and acetylcholine was linear within the dosage range studied (Fig. 1). It appears, however, that there is a maximum for each drug, and that this tissue is more sensitive to adrenaline than to acetylcholine. No tissue fixation occurred with either of these drugs, and recovery of sensitivity was complete after washing for one minute. The rat seminal vesicle shows the same insensitivity to histamine as do other tissues of this species; doses of histamine as high as 2.2 mg. induced no contractions.

Evaluation of Sympatholytic Activity.—A comparison of the potencies of various sympatholytic drugs is given in Table I. There is no significant difference in the potency of piperoxane when evaluated by the single dose and by the multiple dose methods. Furthermore, ergotamine does not differ significantly in potency from piperoxane. Potency ratio comparisons, based on piperoxane as standard, show the following order of decreasing potency: "Dibenzyline," "Dibozane," "Sy-28," "Hydergine," and ergotamine.

Tissue Fixation of Sympatholytic Drugs.—There is variability in the degree of tissue fixation of the various sympatholytic drugs when they are given at their approximate ED50's and then tested every six minutes (Fig. 2). Drugs from the same chemical series show a similar degree of tissue fixation; the 2-haloalkylamines appear to attach themselves strongly to tissue receptors, whereas the benzo-

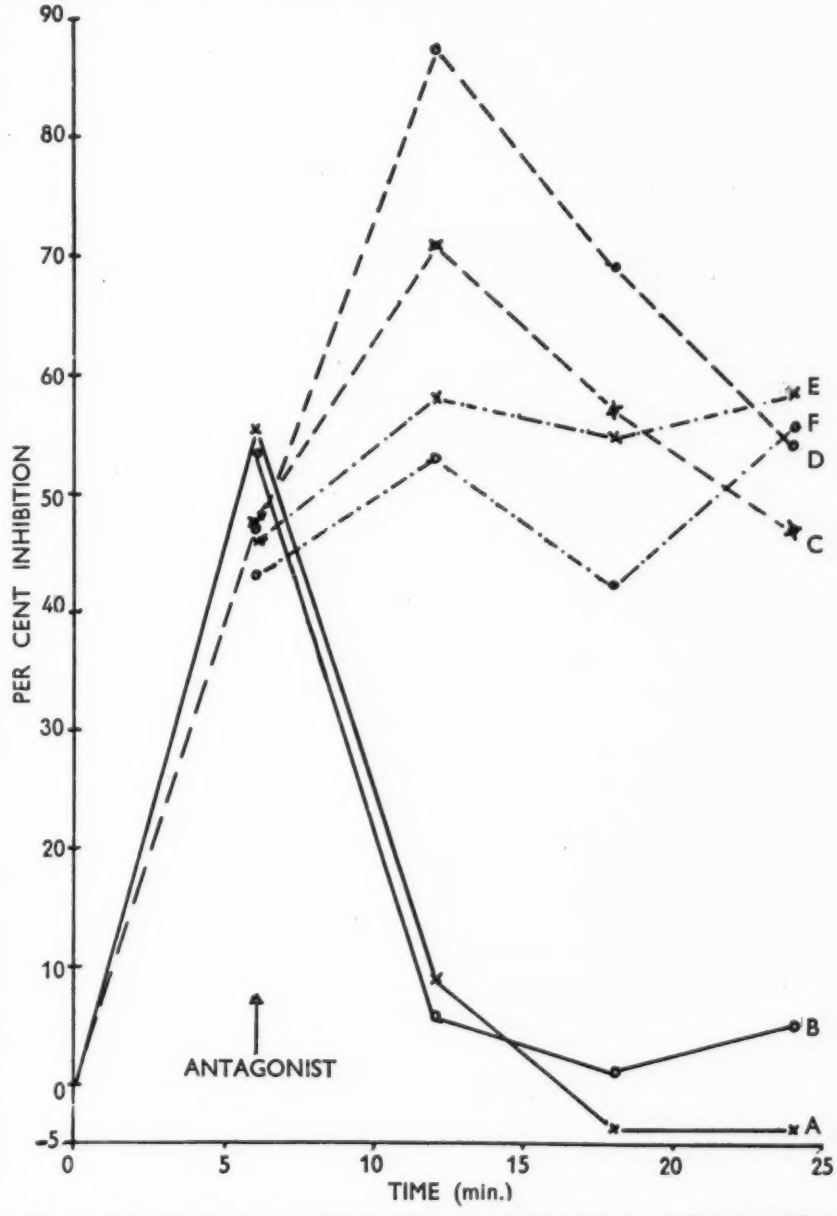

FIG. 2.—Tissue fixation of sympatholytic agents. A, Dibozane 0.5 μg. B, Piperoxane 6.0 μg. C, Hydergine 2.0 μg. D, Ergotamine 3.0 μg. E, Dibenzyline 0.1 μg. F, Sy-28 1.0 μg.

TABLE I

COMPARISON OF SYMPATHOLYTIC ACTIVITY ON RAT'S ISOLATED SEMINAL VESICLE

Drug	Dosage Range µg./15 ml.	ED50 and Range* µg./15 ml.	Slope and Range*	Potency Ratio and Range* µg./15 ml.
Piperoxane hydrochloride 2-(1-piperidylmethyl)-1, 4-benzodioxane HCl	2–10	4·85 (2·98–7·91)	2·43 (1·07–5·84)	Standard
Piperoxane hydrochloride†	1–75	5·15 (2·59–10·3)	3·05 (1·65–5·64)	Equivalent to standard
Ergotamine methanesulphonate	2–5	3·00 (2·42–3·72)	1·74 (1·15–2·62)	" "
"Hydergine"‡	0·8–6·0	1·83 (1·15–2·92)	3·28 (1·37–7·85)	>2·65 (1·35–5·2)
"Sy-28" *N*-(2-bromoethyl)-*N*-ethyl-1-naphthalene-methylamine HBr	0·6–2·0	1·18 (0·8–1·74)	2·5 (1·05–5·95)	>4·11 (2·2–7·65)
"Dibozane" 1, 4 bis(1, 4-benzodioxane-2-ylmethyl)-piperazine	0·15–1·5	0·47 (0·29–0·75)	3·38 (1·5–7·6)	>10·32 (5·2–20·5)
"Dibenzyline hydrochloride" *N*-(phenoxy*iso*propyl)-*N*-benzyl-2-chloroethylamine HCl	0·05–0·2	0·098 (0·06–0·15)	3·1 (0·97–9·9)	>49·5 (25·4–96·5)

* All values at 95% confidence limits.
† Multiple dose per preparation experiment.
‡ An equi-mixture of dihydroergocornine, dihydroergocristine, and dihydroergokryptine.

dioxanes can be removed readily by washing. The ergot alkaloids occupy an intermediate position and they might have been removed from their tissue attachment by washing for a longer period.

DISCUSSION

The relationship between log-concentration of acetylcholine and the response of the isolated rat seminal vesicle is similar to that obtained with the frog's isolated ventricle and rectus abdominis preparations (Clark, 1926, 1927). Such a relationship also exists for the response to adrenaline and is in accord with results obtained on the rabbit's isolated uterus (Gaddum, 1926) and the cat's nictitating membrane (Rosenblueth, 1932; and Bacq and Fredericq, 1935). A sigmoid curve always results although a good portion of this is essentially linear (Fig. 1). The type of curve obtained, and the rapid restoration of sensitivity to both acetylcholine and adrenaline on washing, suggests that a chemical reaction occurs between the drugs and surface receptors like that postulated for the frog's heart by Cook (1926), and for arterial strips by Wilkie (1928). The sensitivity of the rat's seminal vesicle preparation to both acetylcholine and adrenaline is within the same range as that of the guinea-pig's seminal vesicle (Brugger, 1945; Rothlin and Brugger, 1945; Meier, 1950; and Stone and Loew, 1952). The rat preparation has the advantage of being insensitive to histamine. This insensitivity could be exploited in the assay of mixtures containing all three drugs.

The potencies of the various sympatholytic agents as determined with the rat's seminal vesicle preparation agree favourably with those obtained by other procedures (Nickerson, 1949). However, the high potency of dibozane could not be predicted upon the basis of its chemical structure, because the related compound piperoxane had the lowest potency of all of the drugs evaluated. On the other hand, Marsh (1953) found dibozane to be one of the most potent sympatholytic drugs available.

In considering the degree of tissue fixation of sympatholytic agents (Fig. 2), it is necessary to consider tissue penetration, lipoid solubility, and direct chemical reactions with either tissue receptors or cell surfaces or both. The majority of cell membranes behave as though they are composed of two molecular layers of lipoid with a monomolecular layer of protein on either side (Davson and Danielli, 1943). The benzodioxanes have a greater water solubility than either the ergot alkaloids or the 2-haloalkylamines, and water solubility would be one of the factors determining the ease of removal from the cell surface in the absence of direct chemical combination with cell surface constituents. On the other hand, the lipophilic nature of the 2-haloalkylamines would assist their penetration of the cell membrane whereas their hydrophobic characteristics would delay their removal. However, the greatest factor determining tissue fixation is direct chemical reaction with tissue receptors and the 2-haloalkylamines have been shown to form immonium and vinyl intermediates which combine with cell constituents so as to give a prolonged blocking action (Nickerson and Nomaguchi, 1951). Such considerations would support the contention that the 2-haloalkylamines are firmly attached to the tissue receptors of the rat seminal vesicle, as illustrated in Fig. 2.

SUMMARY

1. The rat's isolated seminal vesicle gives a linear log-dose response curve to both adrenaline and acetylcholine. It is more sensitive to adrenaline than to acetylcholine and is completely insensitive to large doses of histamine.

2. Sympatholytic potency can be estimated on the rat's seminal vesicle; the results agree with

those obtained by other methods. The following order of decreasing potency of the various drugs tested was obtained: " Dibenzyline," " Dibozane," " Sy-28," " Hydergine," ergotamine, and piperoxane.

3. It is possible to determine the degree of drug fixation on the tissue with the method. The 2-haloalkylamines have the greatest degree of tissue fixation and the benzodioxanes the least, with the ergot alkaloids occupying an intermediate position.

The authors wish to thank Sandoz Pharmaceuticals for the ergot alkaloids, Smith, Kline and French Laboratories for the " Dibenzyline," Parke-Davis Company for the " Sy-28," and McNeil Laboratories, Inc., for the " Dibozane " used in this investigation.

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DRUGS WHICH ANTAGONIZE 5-HYDROXYTRYPTAMINE

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A specific antagonist for 5-hydroxytryptamine (HT) might be valuable in various ways. It might provide evidence for the identification of HT in tissue extracts. It might help those who are interested in other pharmacologically active substances by suppressing interference due to HT. It might also have direct actions of its own which would provide a clue to the physiological function of HT which is found in tissues. This paper describes part of the search for such an antagonist. Some of these results were communicated to the British Pharmacological Society in July, 1952.

METHODS

Rats' uteri were prepared by the subcutaneous injection of stilboestrol (10 μg. per 100 g. wt.) the day before the experiment. Ovariectomy (Erspamer, 1952a) did not seem to be necessary. The uteri were suspended in a 2 ml. bath at 30° C. and the solution was that recommended by Gaddum, Peart, and Vogt (1949). Their contractions were recorded with a lever on a smoked drum with a magnification of about 5. Drugs were added to the bath in small measured volumes, and the concentrations recorded here were the final concentrations in the bath. Either acetylcholine (ACh) or carbachol was given in alternate doses to control the specificity of the effect of antagonist drugs.

Isolated pieces of guinea-pig's ileum were suspended in a 2 ml. bath containing Tyrode's solution maintained at 37° C. and the movements of the longitudinal muscle recorded with a lever. The portion of the ileum adjacent to the caecum was found to be most sensitive to HT. Histamine and choline esters were used as control drugs.

Isolated rabbits' ears were perfused at room temperature through a cannula in the central artery. A special injection cannula, as described by Gaddum and Kwiatkowski (1938), was interposed between the reservoir containing the perfusion fluid and the arterial cannula. The drugs were injected through the rubber cap of this cannula. Two reservoirs were connected with the injection cannula, one containing the ordinary perfusion fluid and the other containing the antagonist. The preliminary steps of dissection and cannulation of the artery were the same as described by Page and Green (1948). The ear was placed on a draining plate and the perfusate from the cut surface was led to a small collecting funnel which led into a capillary. This capillary end was connected to a drop-timer (Gaddum and Kwiatkowski, 1938). In the tracings, the height of the record measures the interval between successive drops. The perfusion fluid was that recommended by Page and Green (1948) for the study of serum vasoconstrictor, and had the following composition (g./l.):

NaCl, 8.2; KCl, 0.84; $CaCl_2,2H_2O$, 0.04; $MgCl_2,6H_2O$, 0.06; $NaHCO_3$, 0.4; glucose, 1. To each litre was added 10 ml. of phosphate buffer containing 4 parts of 1 M-K_2HPO_4 to 1 part of 1 M-KH_2PO_4.

When this solution was used, the ear was much more sensitive to vasoconstrictor substances than when Locke's solution was used. The ear was always more sensitive on the next day after being kept overnight in the refrigerator. Adrenaline was used as a control drug.

We are indebted to various manufacturers and others for preparations of the following drugs—5-hydroxytryptamine creatine sulphate (Messrs. Upjohn), tryptamine hydrochloride (British Drug Houses), various ergot alkaloids (Sandoz Products), piperoxane (May & Baker, Ltd.), mescaline sulphate (Roche Products, Ltd.), gramine (L. Light & Co., Ltd.), dibenamine (Smith, Kline & French Research Laboratories), cinobufotenine (Dr. K. K. Chen). Doses are given in terms of the bases.

RESULTS

HT was found to cause contractions of the uterus, duodenum, and colon of rats; the uterus, duodenum, and jejunum of rabbits; and the uterus, duodenum, jejunum, and ileum of guinea-pigs. It caused vasoconstriction in the perfused ears of rabbits. The most sensitive of these tissues were the rat's uterus (Erspamer, 1952a), the guinea-pig's ileum (Gaddum, 1953a; Robertson, 1953; Rocha e Silva, Valle, and Picarelli, 1953), and the rabbit's ear (Rapport, Green, and Page, 1948), all of which have been used before for experiments with HT.

There is evidence that some of the actions of HT and tryptamine are due to combination with

special receptors, which are not identical with the receptors for histamine, adrenaline, or ACh, and which have been called tryptamine-receptors (Gaddum, 1953a). These other drugs all have effects like those of HT on some tissues but not on all tissues. Histamine and adrenaline do not stimulate the rat's uterus, adrenaline does not stimulate the guinea-pig's ileum, and ACh causes vasodilatation rather than vasoconstriction in the freshly prepared rabbit's ear (Burn and Robinson, 1951).

In every case a test of specificity was carried out by comparing the effect of HT with that of a suitable dose of some other drug before and after the addition of the antagonist. When the effects of both drugs were depressed the antagonism was considered unspecific and unimportant. Such effects may be caused by depression or poisoning of the contractile mechanism. The statement that an antagonism was specific means that the effect of HT was depressed while the effect of the control drug was not. This test does not prove that HT and its antagonist are competing for the same receptor, but does exclude some cases in which they are not.

Guinea-pig's ileum can be specifically desensitized to HT by exposure to high concentrations of this drug or tryptamine (Gaddum, 1953a ; Rocha e Silva *et al.*, 1953). Similar results may sometimes be obtained with the rat's uterus. In one experiment, for example, HT (75 μg./l.) and tryptamine (7,500 μg./l.) caused effects similar to those of carbachol (750 μg./l.). A high concentration of HT (15,000 μg./l.) was then added to the bath for 43 min. This caused strong contractions, which disappeared after 13 min. The muscle now gave no response to HT or tryptamine, but the effect of carbachol was unchanged. This desensitization persisted for 15 min. after the high concentration of HT had been removed ; the muscle then again gave a response to tryptamine. In some experiments the effect was obscured by the persistence of muscular contractions in the presence of HT ; the inhibitory effect of high concentrations of HT was much less clearly shown on the rat's uterus than on the guinea-pig's ileum. It would not be easy to do similar experiments with the rabbit's ear, because large doses of HT stop the flow completely.

Specific antagonisms are commonly due to the competitive action of drugs similar in structure to the active drug, and tests have therefore been made with a number of compounds which, like HT, contain an indole nucleus. Most of these were specially prepared by Messrs. Glaxo, and the results will be published in a separate paper.

Gramine (3-dimethylaminomethyl-indole) acted as the prototype for a number of active synthetic anti-HT compounds, and its effect on the rat's uterus is shown in Fig. 1. It abolished the response to HT without affecting the response to carbachol. Erspamer has observed the same effect (1954). In a concentration of 10 mg./l. it decreased the sensitivity of the rabbit's ear to HT by more than 2,000-fold and that to adrenaline by about 15-fold. On the other hand it had no definite effect on the response of the guinea-pig's ileum.

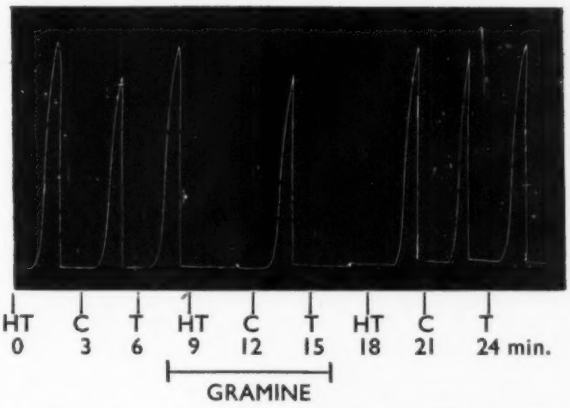

FIG. 1.—Rat's uterus. 2 ml. bath. Interval 3 min. HT, 5-hydroxytryptamine 20 ng. C, Carbachol 100 ng. T, Tryptamine 4,000 ng. Gramine (20 μg.) specifically inhibited tryptamine receptors.

Ergot Alkaloids.—Tryptamine forms part of the molecule of lysergic acid, certain derivatives of which are very active antagonists of HT on the rat's uterus and the rabbit's ear.

The antagonism of ergotoxine and tryptamine was described by Laidlaw (1911) and that of ergotamine and α-methyltryptamine by Seki (1929). Heymans, Bouckaert, and Moraes (1932) found that ergotamine antagonized the vasoconstrictor action of defibrinated blood, which was presumably due mainly to HT. Gaddum, Peart, and Vogt (1949) obtained similar results with dihydroergotamine and made the additional observation that suitable concentrations of this drug suppressed the response of the rabbit's ear to cat's plasma without altering the response to adrenaline. This fact seemed surprising at the time but is now explained, since the response to HT can also be suppressed without altering the response to adrenaline.

The results recorded below confirm and extend those obtained by various workers who have studied the antagonism of HT and ergot alkaloids during recent years (Erspamer, 1952a ; Woolley and Shaw, 1953 ; Fingl and Gaddum, 1953 ; Page and McCubbin, 1953).

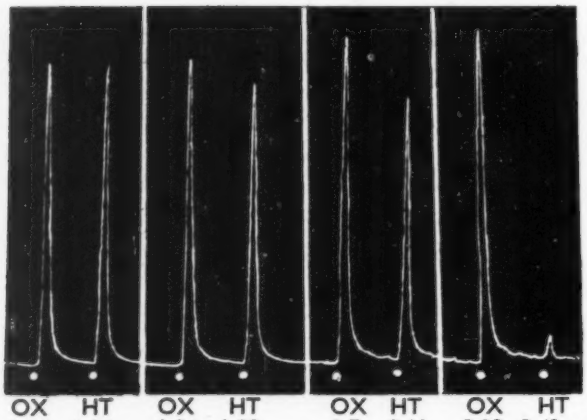

FIG. 2.—Rat's uterus. 3 ml. bath. OX, oxytocin 30 mu./l. HT, 5-hydroxytryptamine 16 μg./l. LSD specifically inhibited tryptamine receptors.

The most active and specific of these derivatives which has been tested is lysergic acid diethylamide (LSD). A preliminary account of this action has already been published (Gaddum, 1953b). Fig. 2 shows the result of an experiment on the rat's uterus in which LSD (0.3 μg./l.) caused a small decrease in the effect of HT (16 μg./l.) while a higher concentration (3 μg./l.) inhibited the effect almost completely. Control doses of oxytocin (30 mu./l.) gave effects which were not altered by the LSD. When the LSD was removed from the bath and the administration of the other drugs continued, the response to HT returned almost to its original value in about an hour. A concentration of 0.6 μg./l. of LSD reduced the effect of HT (15 μg./l.) so that after 10 min. the dose of HT had to be doubled to reproduce the original effect. It was thus estimated that the pA_2 (10 min.) was about 8.7 (Schild, 1947). This indicates that LSD had about the same activity in this experiment as atropine had when tested by Schild as an antagonist for ACh on guinea-pig's intestine. When the LSD was left in the bath its action continued to increase for at least 20 min.

Various derivatives of lysergic acid have been compared for their effects on the rat's uterus as antagonists of HT and placed in the following descending order of potencies—lysergic acid

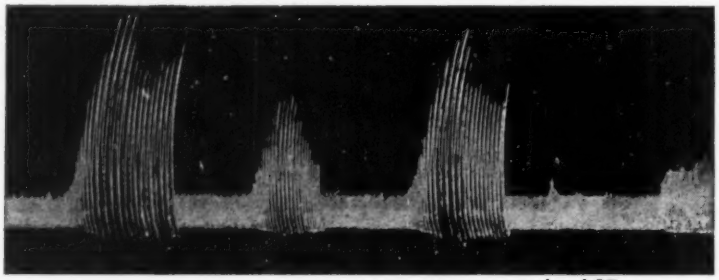

FIG. 3.—Rabbit's ear perfused. Height of record shows interval between drops. HT, 5-hydroxytryptamine. AD, adrenaline (doses in ng.). LSD (10 μg./l.) specifically inhibited response to HT.

diethylamide, dihydroergotamine dihydroergocornine, and dihydroergokryptine. Ergotamine, ergotoxine, and ergometrine generally caused much spontaneous activity in the concentrations used, but in all cases there was some evidence of a fall in the response to HT in conditions where there was no change in the response to a choline ester. Dihydroergocristine caused marked inhibition, but this was not specific.

Some of these substances were also tested on the rabbit's ear. With this tissue it is possible to compare their actions against HT and adrenaline, both of which cause vasoconstriction; whenever this comparison has been made the response to HT has been depressed more than the response to adrenaline.

Fig. 3 shows the results of an experiment where HT (6 ng.) had more vasoconstrictor action than adrenaline (1 ng.). LSD was then perfused in a concentration of 10 μg./l., and this abolished the constrictor effect of HT even when the dose was increased to 2,000 ng. At the same time the response to adrenaline showed a gradual increase.

Fig. 4 shows the results of a similar experiment with dihydroergotamine. A concentration of 20 μg./l. of this drug abolished the response to HT and greatly diminished that to adrenaline, but when the doses were increased adrenaline was again effective. It was evident that dihydroergotamine was more active as an antagonist to HT than as an antagonist to adrenaline, but its action was not so specific as that of LSD.

In a concentration of 20 μg./l. ergotamine did not produce any significant inhibition of the response of the rabbit's ear to HT. In a concentration of 40 μg./l. the responses to both HT and adrenaline were abolished, but a four-fold dose of adrenaline was effective, while a 100-fold dose of HT was not. Ergometrine, even in a concentration of 100 μg./l., had no significant effect on the response to HT or adrenaline.

The antagonism between HT and the ergot alkaloids on the guinea-pig's ileum is comparatively feeble and less clearly specific. In a concentration of 100 μg./l. LSD reduced the response to HT by 50%, but much higher concentrations (10,000 μg./l.) appeared to have no more effect. The responses to histamine and ACh were not altered in these experiments.

Low concentrations of dihydroergotamine had no effect, but when higher concentrations (2,500–25,000 μg./l.) were used the response to HT was depressed. In these conditions the response to histamine was also depressed, sometimes even more than that to HT. Similar results were obtained with rabbit's duodenum.

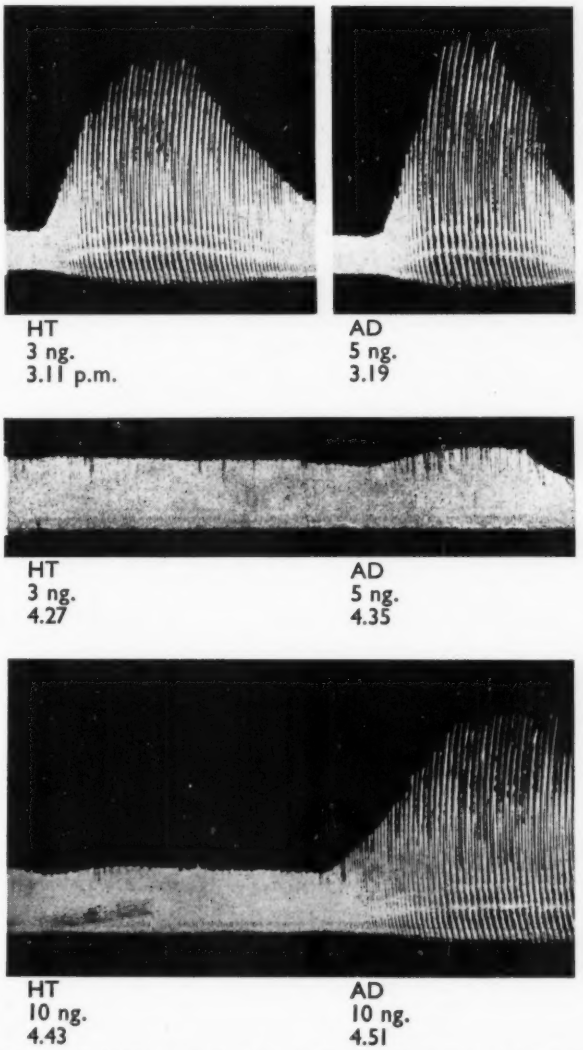

FIG. 4.—Rabbit's ear perfused as in Fig. 3. Top: control doses of 5-hydroxytryptamine (3 ng.) and adrenaline (5 ng.). From 3.25 dihydroergotamine (20 μg./l.) was continuously present in the perfusion fluid. Both drugs antagonized, but HT more than adrenaline.

Piperoxane (933F, piperidylmethylbenzodioxane). —Piperoxane is known as an antagonist of adrenaline, but it had no antagonistic action to HT on the rat's uterus (50 mg./l.) or the rabbit's ear (400 μg./l.). This result confirms those of Erspamer (1953) and Fingl and Gaddum (1953). Fig. 5 shows the results of an experiment in which HT was first compared with adrenaline and found to be about 1.5 times as active. The perfusion fluid was then replaced by a similar fluid containing piperoxane (400 μg./l.). This abolished the

response to adrenaline without any definite effect on the response to HT. It is clear that antagonisms on the rabbit's ear to adrenaline and HT may vary independently. LSD is more active against HT and piperoxane is more active against adrenaline.

Dibenamine (*NN*-dibenzyl-β-chloroethylamine) is known as an antagonist of adrenaline with a very prolonged action. It also has some antihistamine activity (Nickerson, 1949; Fleckenstein, 1952). It antagonized the action of HT on all of the three tissues used in the present experiments.

A concentration of 5 μg./l. of dibenamine greatly reduced the response of a rat's uterus to HT without altering the response to ACh. The effect developed gradually for an hour or more. A concentration of 100 μg./l. abolished the response to HT and slightly reduced that to ACh. With higher concentrations the response to HT was immediately, completely, and irreversibly blocked.

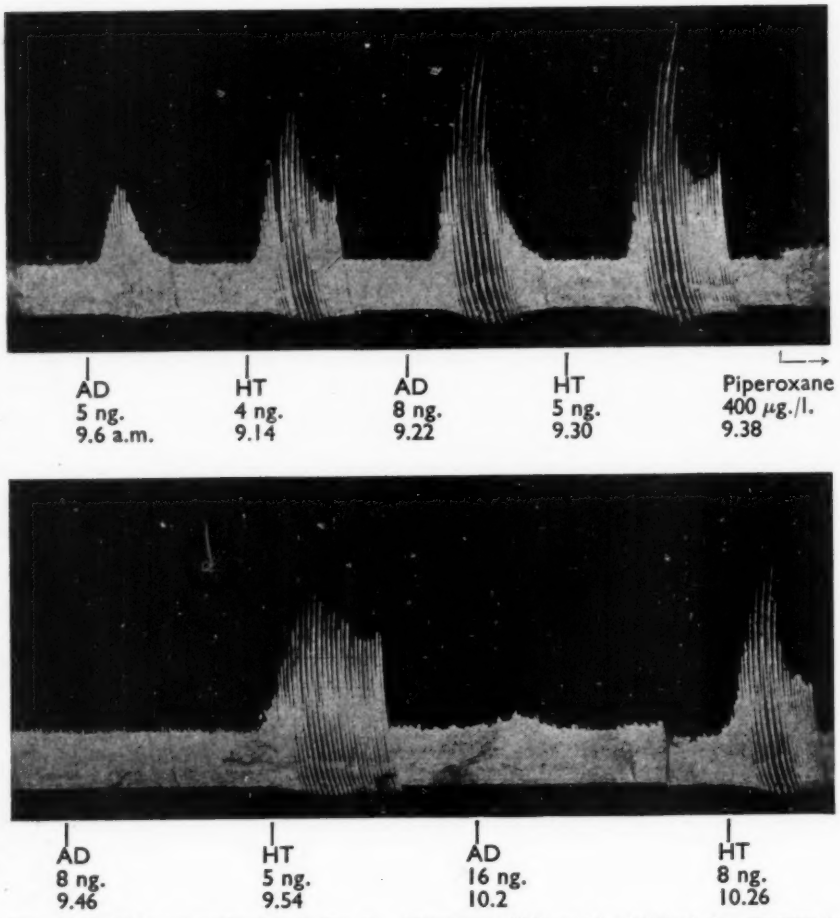

FIG. 5.—Rabbit's ear perfused as in Fig. 3. Piperoxane (400 μg./l.) abolished the response to adrenaline without much effect on the response to HT.

The receptors in the rabbit's ear were less sensitive to dibenamine. A concentration of 100 μg./l. caused 75% inhibition of the response to adrenaline and 64% inhibition of the response to HT. Both effects persisted when the dibenamine was washed away.

The guinea-pig's ileum was less sensitive than the rabbit's ear. Dibenamine (1,000 μg./l.) reduced the responses to histamine and HT about equally without affecting that to ACh. When the dibenamine was washed away the response to HT returned, but not that to histamine. Dibenamine was thus found to be a powerful antagonist for HT on the rat's uterus (Erspamer, 1952a, 1953). On the other two tissues its action against HT was feebler, and less than its actions against adrenaline and histamine (Fingl and Gaddum, 1953).

Inhibitors of Amine Oxidase.—In low concentrations ephedrine potentiates some of the actions of adrenaline and in higher concentrations it antagonizes them (Gaddum and Kwiatkowski, 1938). The first action has been attributed to the inhibition of amine oxidase, which ephedrine is known to cause, and since HT is destroyed by amine oxidase (Blaschko, 1952; Freyburger, Graham, Rapport, Seay, Govier, Swoap, and Vander Brook, 1952) it was thought possible that some of its effects might be increased by ephedrine and allied drugs. Experiments with ephedrine and amphetamine on the rat's uterus and the guinea-pig's ileum did not show any significant action of this kind, but it was found that the response of the perfused rabbit's ear to HT was greatly increased when ephedrine (10 mg./l.) was present in the perfusion fluid. In these experiments the response to adrenaline was actually depressed by this concentration of ephedrine (Fig. 6). The potentiation of the effect of HT is presumably due to the inhibition of amine oxidase. The inhibition of the response to adrenaline by higher concentrations of ephedrine has been attributed to blockade of the adrenaline

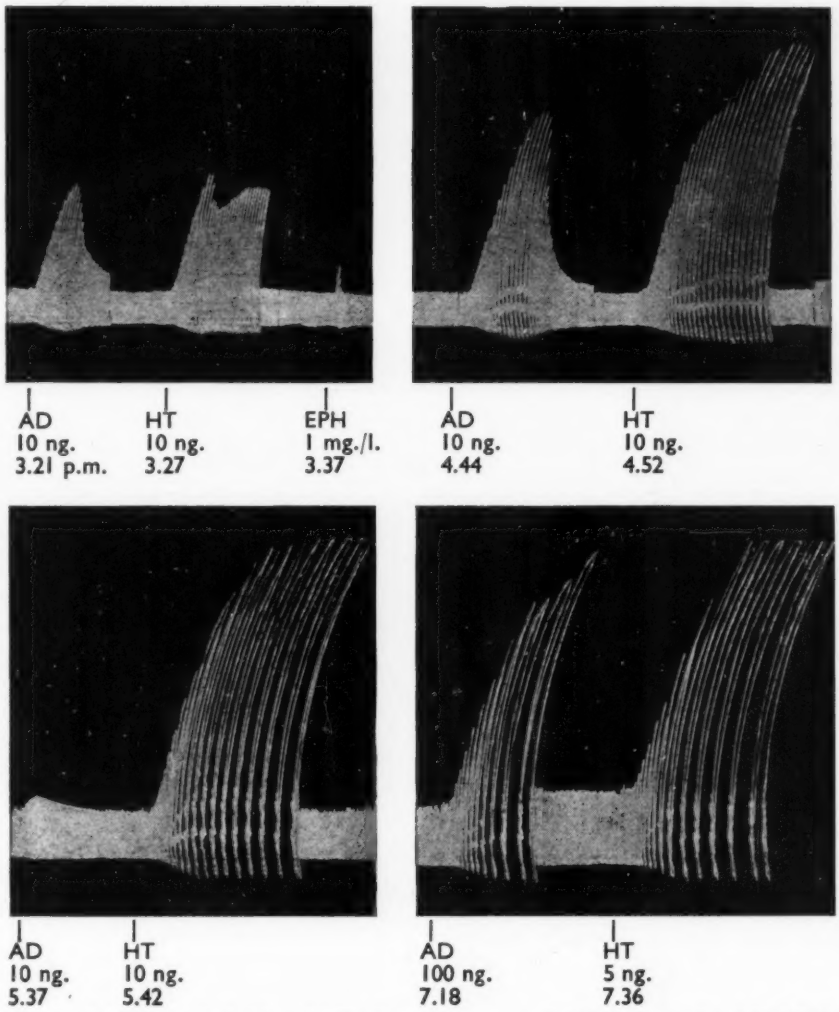

FIG. 6.—Rabbit's ear perfused as in Fig. 3. Doses in ng. Ephedrine (1 mg./l.; 3.37–5.7) increased the responses to adrenaline (AD) and 5-hydroxytryptamine (HT). In a higher concentration (10 mg./l.; 5.7–7.50) ephedrine diminished the response to adrenaline, but increased the response to HT still more.

receptors (Gaddum and Kwiatkowski, 1938). The tryptamine receptors do not appear to be blocked by the concentrations of ephedrine used in these experiments.

The theory that this potentiation of the response to HT is due to the inhibition of amine oxidase was confirmed by the results of one experiment with choline-*p*-tolyl ether bromide (Brown and Hey, 1952; Hey, 1952). This drug, which is known to inhibit amine oxidase, was added to the fluid perfused in a rabbit's ear in a concentration of 1 mg./l. This increased the effects of both adrenaline and HT; a higher concentration (10 mg./l.) decreased both effects.

Cocaine.—Cocaine is not only a local anaesthetic but also an inhibitor of amine oxidase (Philpot, 1940). Reid and Rand (1952) found that it increased the effect of HT on a strip of sheep's carotid artery, but reported no experiments to test whether this was a specific effect, or an unspecific increase in the contractile power of the muscle. In our experiments cocaine did not show any effects which could be attributed with confidence to the inhibition of amine oxidase. In a concentration of 5–10 mg./l. it increased the effects of both HT and ACh on the rat's uterus. When it was washed away the effects gradually returned to their original level. Lower concentrations had no significant action. A higher concentration (50 mg./l.) temporarily inhibited the effects of both HT and ACh (Fig. 7).

The action of HT on the rabbit's ear was not significantly changed by cocaine (10 mg./l.), although the action of adrenaline was much increased in the same experiment. It has been suggested that the effect of cocaine on the response to adrenaline is due to the inhibition of amine oxidase (Philpot, 1940; Blaschko, 1952). If this is so, it is perhaps surprising that the cocaine did not increase the effect of HT in this experiment. It might be suggested that the effect of HT on the rabbit's ear does not depend on amine oxidase, but if so the potentiating action of ephedrine cannot be explained in the way that has been suggested above.

Cocaine (5–10 mg./l.) inhibited the response of the guinea-pig's ileum to HT reversibly without much effect on the responses to histamine and ACh. The response to nicotine was inhibited to the same extent as the response to HT. This inhibition has been observed by others (Sinha and West, 1953; Rocha e Silva *et al.*, 1953) and explained on the theory that HT acts on the nervous tissues in the intestine and that cocaine inhibits its action by paralysing these nerves.

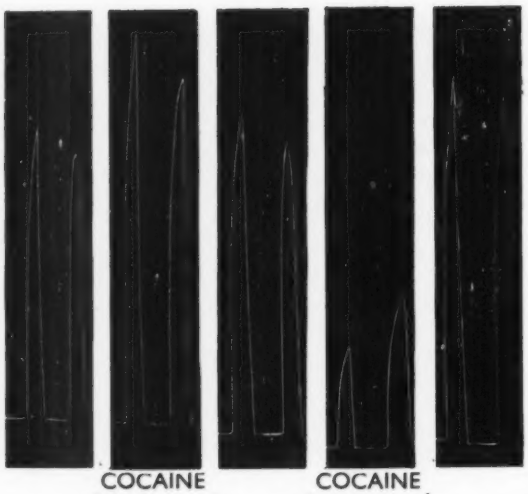

FIG. 7.—Rat's uterus. 2 ml. bath. AC, acetylcholine, 500 ng.; HT, 5-hydroxytryptamine, 10 ng. Cocaine (10 mg./l.; 5–17 min.) increased both effects. A higher concentration of cocaine (50 mg./l.; 37–57 min.) decreased both effects.

The actions of cocaine on these three tissues were thus different. The response of the rat's uterus was unspecifically increased by low concentrations and unspecifically decreased by higher concentrations. The response of the rabbit's ear was unaffected and the response of the guinea-pig's ileum was inhibited, like the response to nicotine, before the responses to histamine or ACh. The generalization of Sinha and West (1953), that local anaesthetics antagonize the actions of HT, does not give all the facts.

Atropine.—In concentrations of 5–100 μg./l. atropine completely abolished the effect of carbachol (50 μg./l.) on the rat's uterus, while leaving the effect of HT unchanged. A higher concentration (1,000 μg./l.) diminished the effect of HT by 50%.

Atropine (5 μg./l.) abolished the response of the guinea-pig's ileum to carbachol (5 μg./l.) but not that to HT (50 μg./l.) (Fig. 8). Higher concentrations of atropine (100 μg./l.) diminished the effect of HT and abolished the effect of ACh (3–5 μg./l.) while leaving that of histamine (3–5 μg./l.) unchanged. These results confirm those of previous workers (Gaddum, 1953a; Rapport and Koelle, 1953; Robertson, 1953; Rocha e Silva *et al.*, 1953). Atropine inhibits the effect of HT on this tissue more than that of histamine, but less than those of choline esters. The actions of atropine on the guinea-pig's ileum are thus similar to those on the rat's uterus, but there was less difference between the doses effective against HT and ACh.

Atropine (100–1,000 μg./l.) had no action on the effect of HT on the rabbit's ear.

Antihistamines.—Experiments were done with mepyramine and diphenhydramine. The first is one of the most specific antihistamines, and the second is less specific since it has some action against ACh (Schild, 1947).

The effect of HT on the rat's uterus was partially blocked by either drug in a concentration of 100 μg./l. and completely blocked by 1,000 μg./l. With diphenhydramine the response to ACh was inhibited to approximately the same extent as the response to HT.

Mepyramine (1,000 μg./l.) had little or no effect on the response of the guinea-pig's ileum to HT or ACh, though a concentration of 5 μg./l. was sufficient to abolish the response to histamine. Diphenhydramine (25–1,000 μg./l.) produced partial inhibition of the responses to both HT and ACh, and abolished the response to histamine almost completely.

These two antihistamines thus produced much the same effect on the responses of both tissues to HT as on their responses to ACh. The experiment with the guinea-pig's ileum provides another example confirming the generalization that mepyramine is a more active and more specific antihistamine than diphenhydramine.

These results are in general agreement with those of Reid and Rand (1951, 1952), Rapport and Koelle (1953), and Erspamer (1952b).

Inactive or Unspecific Antagonists.—Hexamethonium (10 mg./l.) inhibited the effect of

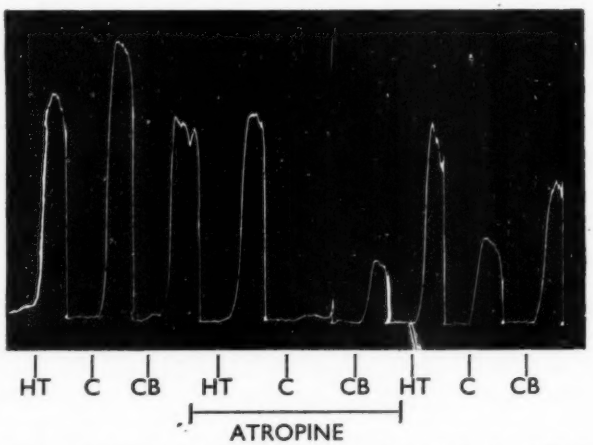

FIG. 8.—Guinea-pig's ileum. 2 ml. bath. Interval 3 min. HT, 5-hydroxytryptamine 100 ng. C, carbachol 10 ng. CB, cinobufotenine (*N*-trimethyl-5-hydroxytryptamine) 1,200 ng. Atropine (10 ng. during time signal) suppressed the response to carbachol but not that to HT. With CB the effect was intermediate.

nicotine on the guinea-pig's ileum without altering the effect of HT. This confirms the results of Robertson (1953) and Rocha e Silva *et al.* (1953).

Mescaline (50 mg./l.) did not affect the response of the rat's uterus to HT. Mescaline is thought by some to resemble lysergic acid diethylamide in its effects on the central nervous system. These two drugs might therefore have been expected to have similar effects as antagonists of HT, but this was not the case.

Yohimbine (5 mg./l.) markedly depressed the response of the rat's uterus to HT and tryptamine. The response to ACh was also depressed, though less, and the antagonism was thus not very specific (cf. Erspamer, 1953). The antagonism between yohimbine and HT on isolated strips of carotid artery has been described by Reid and Rand (1952), Woolley and Shaw (1953), and Shaw and Woolley (1953). These authors obtained effects with concentrations as low as 0.1 mg./l., but give no evidence that the inhibition was specific.

Eserine in low concentrations (100–1,000 μg./l.) caused a small increase in the response of the rat's uterus to HT and higher concentrations sometimes caused a decrease, but the effects were feeble.

Although both yohimbine and eserine contain an indole ring there is as yet no evidence that either of them is a specific antagonist of HT.

Discussion

Experiments with specific antagonists confirm the conclusion that HT produces some of its effects by acting on specific receptors. With mepyramine, piperoxane, and atropine it is possible to abolish the effects of histamine, adrenaline, and ACh respectively, without altering the effects of HT. Higher concentrations of these drugs may antagonize HT, but this does not invalidate the evidence given by the experiments with low concentrations, which show that some at least of the effects of HT are due to combination with receptors which are not identical with the receptors for histamine, adrenaline, or ACh.

The drugs which do antagonize HT have given different results when tested on different tissues. LSD is a very active and specific antagonist for HT in experiments on the rat's uterus or the rabbit's ear, but had little effect in experiments on the guinea-pig's ileum even when high concentrations were used. Other ergot alkaloids and gramine gave similar though less striking results. These drugs were more effective on the rat's uterus and the rabbit's ear than on the guinea-pig's ileum, but the reverse is true of atropine, cocaine, or excess of HT itself.

Other workers (Robertson, 1953 ; Rocha e Silva *et al.*, 1953) have obtained similar results with the last three drugs on guinea-pig's ileum, and have suggested a theory to account for them. The effect of HT on this tissue resembles that of nicotine in that both effects are blocked by similar concentrations of atropine and cocaine. It is therefore suggested that HT acts, like nicotine, at some point in the nervous tissues in the intestine. Hexamethonium, or sufficient excess of nicotine, blocks the response to small doses of nicotine without blocking the response to HT. These facts are explained on the theory that HT acts on the postganglionic nerve fibres whereas nicotine acts on the nerve cells.

This theory does not provide a satisfactory explanation of the observation of Rocha e Silva *et al.* (1953), that when the guinea-pig's ileum is desensitized to HT by excess of HT itself, it still gives a normal response to nicotine. If the site of action of HT is peripheral to that of nicotine and is paralysed, it is surprising that the response to nicotine is normal. Nicotine and HT appear to behave similarly to one another but independently ; if the bath contains excess of either of these drugs the muscle gives no response to that drug, but a normal response to the other drug. There is nothing to suggest an anatomical difference between the sites of action of the two drugs. It is possible that the ganglion cells in the intestine contain two types of receptor, one of which is stimulated by ACh or nicotine and inhibited by excess of nicotine or hexamethonium, while the other type is stimulated by HT and inhibited by excess of HT. These cells would then be comparable with various plain muscle cells which are believed to have specific receptors for both histamine and ACh. It is also possible that there are two types of cell, one of which is stimulated by nicotine and the other by HT.

Whatever may be the explanation of the behaviour of the guinea-pig's ileum, it is clear that the other two tissues behave differently. The results can be explained on the theory that there are two types of tryptamine receptor:

(*a*) Receptors in the plain muscle of the rat's uterus and rabbit's ear which are easily paralysed by LSD but not so easily paralysed by excess of HT.

(*b*) Receptors in the ganglia of the intestine which are easily paralysed by excess of HT but not easily paralysed by LSD.

If this theory is correct, the relation between LSD and HT is similar to the relation between ACh and atropine. In both cases the receptors in smooth muscle are more easily inhibited by the antagonist, and the receptors in the nervous tissues are more easily desensitized by excess of the active drug.

Summary

1. The effects of 5-hydroxytryptamine (HT) and various possible antagonists were tested on rat's uterus, rabbit's perfused ear, and guinea-pig's ileum.

2. With suitable concentrations of mepyramine, piperoxane, and atropine it is possible to inhibit the effects of histamine, adrenaline, and acetylcholine without altering the effects of HT. This supports the view that HT acts on specific receptors, which have been called tryptamine receptors.

3. The effects of various HT antagonists can be explained on the theory that there are two types of tryptamine receptor. One type is present in the smooth muscle of the uterus and ear and is specifically inhibited by low concentrations of lysergic acid diethylamide (LSD). The second type is present in the nervous tissues in the ileum and is not inhibited by LSD.

4. The effect of HT on the ileum is inhibited by atropine or cocaine in the same way that the effect of nicotine is inhibited. The known facts can be explained on the theory that the ganglia in the guinea-pig's intestine contain two types of receptor, one of which is stimulated by nicotine and the other by HT.

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THE CONCENTRATION OF ADRENALINE IN THE PERIPHERAL BLOOD DURING INSULIN HYPOGLYCAEMIA

BY

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An increase in circulating adrenaline after insulin administration has been shown in the cat and the rabbit by indirect methods based on signs of stimulation of organs which had been deprived of their sympathetic nerve supply (Cannon, McIver, and Bliss, 1924; Abe, 1924). An increased secretion of adrenaline into the adrenal vein was demonstrated during insulin hypoglycaemia in the dog by Houssay, Lewis, and Molinelli (1924). Poll (1925) showed that the adrenal medulla contains but little chromaffin substance after the injection of a large dose of insulin. More recently, quantitative estimations of the amines remaining in the adrenal medulla after injection of insulin have been carried out by several authors (e.g., Burn, Hutcheon, and Parker, 1950; West, 1951; Outschoorn, 1952); a fall was invariably reported. Euler and Luft (1952) have shown that the urinary excretion of adrenaline rises in man after an injection of insulin. The reverse phenomenon, namely a fall in the amount of adrenaline secreted into the adrenal vein of the cat made hyperglycaemic by infusion of glucose, was demonstrated by Dunér (1953).

In contrast to these findings, Weil-Malherbe (1953) and Weil-Malherbe and Bone (1952), using a fluorimetric method of adrenaline estimation in human blood, report a decrease in the adrenaline content of peripheral blood after intravenous insulin. This finding seemed difficult to reconcile with current physiological concepts. Since no direct estimations of the adrenaline content of peripheral blood after the injection of insulin have been made by other methods, it was decided to attempt such estimations by a technique which left very little doubt whether the estimated compound was really adrenaline.

METHODS

The experiments were carried out on two dogs and one human volunteer after an overnight fast.

Eight experiments were performed on the dogs with doses of insulin ranging from 0.2 to 2.1 u./kg.; 0.24 u./kg. was used in the one experiment carried out on the human subject. The insulin was injected intravenously shortly after the collection of a control sample of blood.

Collection of Blood.—No difficulty was experienced in obtaining blood samples free of 5-hydroxytryptamine (see later) in man by collecting blood from the brachial vein into a siliconed syringe containing 500 u. heparin. The same technique was successful in the dog, when single blood samples were drawn from one of the leg veins. In those experiments, however, in which a series of blood samples were required at precise time intervals, a technique had to be chosen which avoided frequent puncturing of the veins and even the slightest degree of clotting. Further, the collection had to be carried out without any emotional disturbance of the dog. The following procedure fulfilled these requirements. The dogs were trained to stand in a Pavlov stand. On the day of the experiment, some 0.1% cinchocaine solution was injected into and under the skin covering one of the saphenous or cephalic veins. A cannula consisting of a bent piece of polythene tubing (inner diameter 1 mm.) carrying several holes near its tip was used. Its end was connected by means of a syringe needle to a long piece of soft rubber tubing leading to a 100 ml. burette clamped about 1 m. above the dog. The whole system was filled with sterile 0.9% NaCl solution containing heparin (500 u./100 ml.). A screw clip on the rubber tubing controlled the flow from the burette. The cannula was inserted into the vein by threading it through a serum needle which had been introduced, pointing distally, into the vein. The serum needle was withdrawn as soon as the polythene cannula was in place. The cannula was secured in position by a swab of gauze and strips of adhesive plaster wound around the leg. To avoid clotting it was essential that the heparinized saline should flow through the polythene cannula while it was being introduced into the vein. Once the cannula was in position, the flow was adjusted to not less than 0.5 ml./min. With this arrangement, samples of blood were obtained by

disconnecting the syringe needle from the polythene tubing, discarding the first millilitres of blood, and collecting 12–15 ml. blood into a siliconed centrifuge tube which contained 600 u. of heparin in 0.1 ml. saline. Slight massage of the foot was sometimes used to accelerate the flow, but this was unnecessary when the blood escaped from the vein at some pressure, as was frequently the case. When enough blood had been collected, the needle leading to the infusion burette was replaced into the polythene tube, the tube was flushed with saline and the slow infusion resumed. The long rubber tubing allowed for a certain amount of movement of the dog's leg without danger to the polythene cannula. Excitement of the dog during sample collection was not noticeable. Burette, tubing, needles, and polythene cannula were all sterilized before use.

The first blood sample was collected 5–10 min. after introduction of the cannula; the insulin was then injected through the rubber tubing, and five further blood samples were collected at intervals. The earliest samples were taken 5 min., and the latest 210 min., after the injection.

Blood sugar estimations were carried out by the method of Hagedorn and Jensen (1923).

Extraction of Adrenaline and Noradrenaline.—The extraction process has been described earlier (Vogt, 1952a). Plasma samples of 5.5 ml. were obtained as rapidly as possible from the heparinized, cooled blood and extracted with acid alcohol. The extracts were purified, adrenaline and noradrenaline separated by paper chromatography, the amines eluted, the eluates evaporated to dryness and taken up in 0.55 ml. H_2O (tenfold concentration of the plasma).

Assays.—Adrenaline was assayed on the rat's uterus stimulated by carbachol (Gaddum and Lembeck, 1949). Before the finally adopted technique of blood collection had been developed, the eluates frequently contained a substance which stimulated the rat's uterus. Its position on the chromatogram, and the disappearance of its stimulating effect after the addition to the uterus bath of dihydroergotamine or lysergic acid diethylamide (Gaddum, 1953), make it extremely likely that this substance was 5-hydroxytryptamine (HT). Though assays of adrenaline in a mixture of adrenaline and HT may be carried out by using the antagonists dihydroergotamine and lysergic acid diethylamide, such assays are not satisfactory, because the mixture has frequently not precisely the same effect on the uterus as the same amount of adrenaline alone. They were thus not relied upon and the results of such assays are not included. The interference of very small quantities of HT can be avoided by choosing uteri which are not oestrous; these may be very sensitive to adrenaline and are fairly insensitive to HT.

It soon became obvious that the quantities of adrenaline occurring in blood were even smaller than had been suspected, and required extremely sensitive uteri for their estimation. Though such uteri (sensitive to 0.03–0.2 ng. adrenaline in a 2 ml. bath) are occasionally found, they are rare, and a method of sensitizing the uterine tissue was therefore sometimes resorted to. This consisted in adding small concentrations of the adrenaline antagonist (see Jang, 1941) dibenzyline (SKF 688A or *N*-phenoxy*iso*propyl-*N*-benzyl-β-chloroethylamine HCl) to the bath; doses of between 0.1 and 0.5 μg. were introduced into the 2 ml. bath for a period of 10 min. and then washed out; after this treatment sensitization to the desired degree was usually obtained. If the lowest concentration does not have the desired effect it is best to increase the dose of dibenzyline very gradually; the responses to carbachol are otherwise likely to become depressed and irregular. Since the latent period of the contraction is prolonged by the drug, it is also advisable to leave the carbachol in the organ bath for a period which is between 10 and 15 sec. longer than the normal time of 40 sec.

No inhibition of the carbachol contraction of the uterus was accepted as being due to adrenaline unless the action disappeared on heating the solution in a stoppered flask for 10 min. in a boiling water bath in the presence of $NaHCO_3$. If no adrenaline was found, a test was made to ensure that no "masking" substances were present by adding known amounts of adrenaline to the eluate and comparing the effect of the mixture with the effect of adrenaline dissolved in saline. These criteria were fulfilled for all the figures given under "Results."

Recovery—In an earlier paper (Vogt, 1952a) recovery had been tested by adding 0.2 μg. of adrenaline and noradrenaline per ml. plasma and extracting 5 ml. of the mixture. The chromatographic procedures had been tested by adding a total of 5 ng. adrenaline per sample. These amounts were too large to be satisfactory for the present work, since the largest amounts of adrenaline found were usually of the order of 0.5–2.0 ng./ml. plasma. Recovery experiments were therefore carried out in which adrenaline and noradrenaline were added to 5.5 ml. plasma, to produce final concentrations, per ml., of 2 and 0.4 ng. adrenaline and of 4 and 1 ng. noradrenaline. Recovery at both dose levels was complete within the limits of the error of the assay; losses, therefore, cannot have amounted to more than 30%. The plasma used for these recoveries did not contain amines in concentrations detected by our methods.

Results

Fig. 1 shows the results of four experiments, carried out on one dog, and in which all blood samples were free of interfering amounts of 5-hydroxytryptamine and could be satisfactorily assayed. In three experiments the concentration of adrenaline in the plasma before the injection of insulin lay between 0.04 and 0.25 μg./l. In the fourth ,no adrenaline ($<$0.06 μg./l. was found, but the adrenaline concentration rose to 0.36 μg./l. 5 min. after the injection of 1.8 u./kg. of insulin.

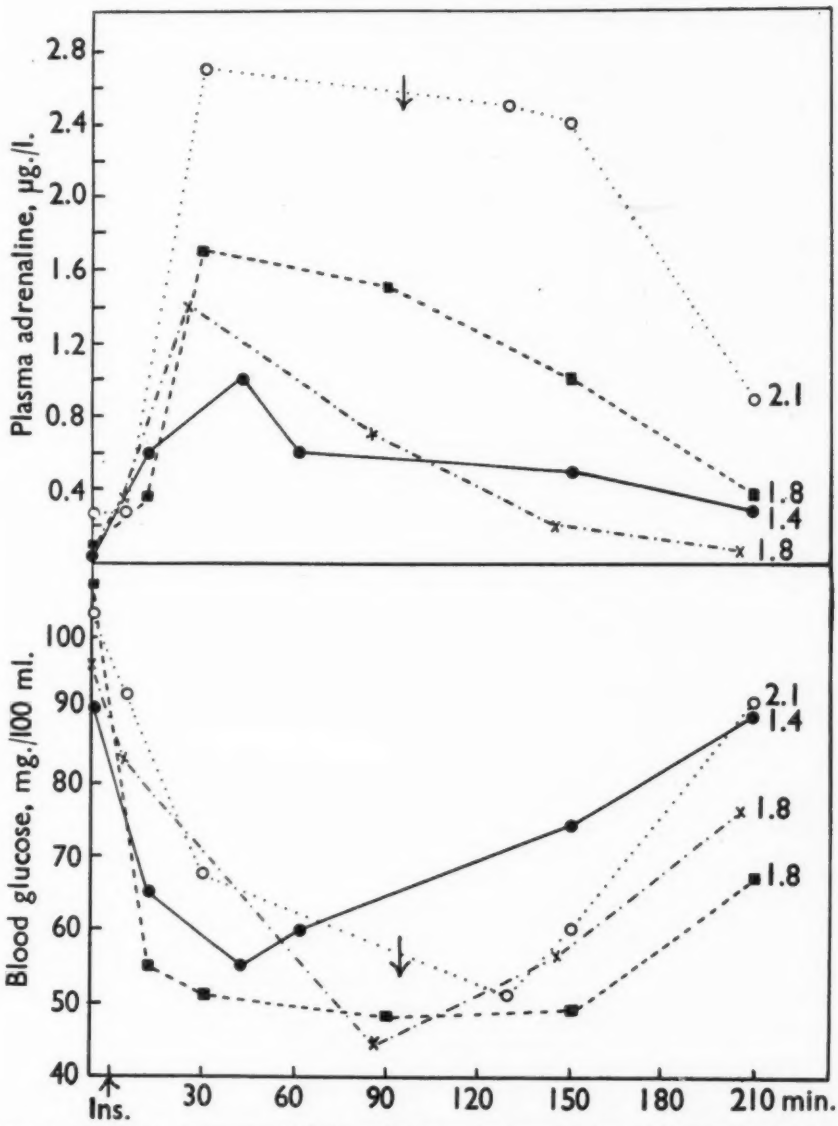

FIG. 1.—Effect of an intravenous injection of insulin on the concentration of adrenaline in plasma. Four experiments on the same dog. Ordinate, upper half, plasma adrenaline in µg./l. Lower half, blood glucose in mg./100 ml. Abscissa, time in min. At the arrow marked 'Ins.' injection of insulin; doses in u./kg. body weight at the end of the curves. At the arrows pointing down, 1 g. glucose was injected intravenously (experiment marked O · · · · · O).

In this dog, the highest levels of plasma adrenaline were attained between 25 and 43 min. after the insulin; in the course of 210 min. the concentration returned towards or to the pre-injection figure. The greatest adrenaline concentrations were observed in the experiment in which the largest dose of insulin was given, despite the fact that the full effect of the insulin was not allowed to develop: the dog was given 1 g. of glucose intravenously 100 min. after the insulin in order to counteract muscular weakness which the animal was showing as a result of the hypoglycaemia.

Experiments carried out on another dog with smaller doses of insulin (0.2–0.8 u./kg.) agreed with the findings of Fig. 1. The data, however, are incomplete, since only three or four out of the six samples collected in each of the four experiments were suitable for assay. In one sample of plasma taken before the injection of insulin, no adrenaline was detectable (<0.5 µg./l.). In two samples obtained 5 min. after the injection, the blood glucose had fallen and the adrenaline had risen sharply to 4.0 and 6.4 µg./l. plasma. Recovery to control levels was quicker than after the larger doses of insulin.

Table I shows the results of a similar experiment conducted in a healthy human subject. The adrenaline concentration in plasma from the cubital vein was <0.06 µg./l. before insulin was injected and rose rapidly to reach 1.8 µg./l. in 45 min. At this time there was some muscular weakness and profuse sweating, and the experiment was terminated by giving glucose. Apart from the smaller dose of insulin required in man to produce the same fall in blood glucose and the same concentration of circulating adrenaline as in the dog, the responses of man and dog were essentially the same.

Noradrenaline estimations were attempted in all the experiments, but none was found. The eluates

TABLE I

EFFECT OF AN INTRAVENOUS INJECTION OF INSULIN (0·24 U./KG.) ON THE CONCENTRATION OF ADRENALINE IN PLASMA OF A HUMAN SUBJECT

Time after Injecting Insulin (min.)	Adrenaline in Plasma (µg./l.)	Blood Glucose (mg./100 ml.)
−1	<0·06	121
5	0·5	75
45	1·8	55

of the noradrenaline paper-strip had a slight pressor effect, equivalent to 0.5 ng. and, occasionally, 1 ng. noradrenaline per ml. plasma, but this effect was heat stable under conditions when noradrenaline, added to the same eluates, was not. It was shown under "Methods" that noradrenaline, added to plasma to produce a concentration of 1 μg./l., was detectable by the assay procedure, so that the concentration of noradrenaline in the plasma of dog and man must have been less than 1 μg./l.

Discussion

The foregoing experiments show that, both in man and in the dog, the concentration of plasma adrenaline rises in the venous blood when hypoglycaemia is produced by insulin. After insulin, the content of adrenaline in the plasma ranged from 0.25 to 6.4 μg./l., and was thus of the same magnitude as that of the 1 to 6 μg./l. found in arterial plasma of rats during a surgical operation under ether (Vogt, 1952b). These observations are difficult to reconcile with those of Weil-Malherbe (1953) and Weil-Malherbe and Bone (1952), who used a chemical method and reported a fall in blood adrenaline in hypoglycaemia. Another discrepancy lies in the "normal" figures for sympathomimetic amines shown by the two methods. According to biological assay, the adrenaline concentration in plasma of the undisturbed conscious dog lay invariably below 0.25 μg./l. and was sometimes less than a quarter of this figure. The one figure obtained in man was $<$0.06 μg./l. The noradrenaline content of plasma was below the threshold of the biological method, which lies at 1 μg./l. Corresponding figures reported by Weil-Malherbe and Bone (1953) are about 1 μg. adrenaline and 5 μg. noradrenaline per l. plasma and are thus at least five times as high as those determined biologically. Since we found the recovery of added amines at these levels perfectly satisfactory, the lower figures can hardly be caused by losses during the extraction process. It is at least conceivable that the chemical method estimates other compounds in addition to the sympathomimetic amines.

Summary

1. The changes in the adrenaline concentration of peripheral venous blood produced by an injection of insulin were followed in the dog and in a human subject.

2. There was invariably a rise in the adrenaline content of the plasma which started a few minutes after the intravenous injection of insulin and lasted for several hours. The rise increased when the dose of insulin was raised. The concentrations observed ranged from 0.25 to 6.4 μg./l. plasma.

3. The method employed for the assay of adrenaline permitted the recovery of adrenaline added to plasma in concentrations of 0.4–2 μg./l.; it was quite adequate for the estimation of the adrenaline concentrations which occurred after the injection of insulin, but not always for the detection of adrenaline before insulin was given; in these samples the concentrations, when above threshold, had a mean of 0.13 μg./l.

4. No noradrenaline was found in any of the plasma samples either before or after the injection of insulin. Since the method allowed the detection of noradrenaline added to plasma in a concentration of 1 μg./l., it follows that the noradrenaline content of all samples was below that figure.

Our thanks are due to Dr. M. Paasonen for acting as subject in our human experiment. One of us (M. V.) is indebted to the Medical Research Council for a grant towards the expenses of this work. We wish to thank Messrs. Smith, Kline and French for a gift of "Dibenzyline." The work was done during the tenure by one of us (M. H.) of a British Council Bursary.

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